GP153: METHODS AND COMPOSITIONS FOR TREATING CANCER

Field of the Invention

The field of the invention is molecular biology and oncology.

Background of the Invention

Surprisingly little is known about the genetic lesions responsible for its genesis, progression, and clinical behavior. For example, in the case of melanoma, although many genes have been implicated in the genesis of this disease, only the INK4a, RAS and BRAF genes have been shown to be true etiologic lesions in a formal genetic sense (Chin et al., Genes Devel. 11:2822-34 (1997); Davies et al., Nature 417:949-54 (2002)). Moreover, advanced malignancy represents the phenotypic endpoint of many successive genetic lesions that affect many oncogene and tumor suppressor gene pathways. Lesions that lead to such a condition may therefore differ from those required to maintain it. Both types of lesions represent rational therapeutic targets in the treatment of cancer.

Summary of the Invention

It has been discovered that a gene designated GP153 functionally complements the K-ras oncogene in an inducible, spontaneous, *in vivo* cancer model (mouse). It has also been discovered that interfering RNAs that target GP153 expression inhibit the growth of certain human tumor cell lines *in vitro*.

Based in part on these discoveries, the invention provides GP153 antagonists that inhibit GP153 gene expression or GP153 protein activity. Antagonists that inhibit GP153 gene expression include an interfering RNA that inhibits the expression of GP153, a GP153 antisense nucleic acid, and an anti-GP153 ribozyme. The sense strand sequences of four exemplary interfering RNAs of the invention include SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6.

Antagonists that inhibit GP153 protein activity include blocking antibodies that bind to a GP153 protein fragment consisting of the extracellular domain, or amino acids 30-704 of SEQ ID NO:1. Other specific examples of GP153 antagonists include, but are not limited to, blocking antibodies that bind to a GP153 protein fragment consisting of amino acids 30-280, 236-488 or 500-704 of SEQ ID NO:1. GP153 antagonists of the invention inhibit tumorigenesis, tumor development, tumor maintenance, tumor recurrence, tumor growth, or the growth of tumor cells *in vitro*.

The invention also provides methods of inducing apoptosis in a cell. The methods include contacting the cell with an effective amount of a GP153 antagonist.

The invention also provides methods of treating a hyperproliferative condition in a mammal, e.g., a human patient. The method includes administering to the mammal an effective amount of a GP153 antagonist. Cancer is an example of such a hyperproliferative condition. Other examples of hyperproliferative conditions are uncontrolled angiogenesis, psoriasis, arteriosclerosis, arthritis and diabetic retinopathy.

In some embodiments, the method of treating a hyperproliferative condition includes administering a second therapeutic agent. The second therapeutic agent

can be, for example, an anti-angiogenic agent, anti-metastatic agent, agent that induces hypoxia, agent that induces apoptosis, or an agent that inhibits cell survival signals. Examples of cancer therapeutics include farnesyl transferase inhibitors, tamoxifen, herceptin, taxol, STI571, cisplatin, fluorocil, cytoxan, and ionizing radiation.

The invention also provides a host cell containing a recombinant DNA construct that includes a GP153-encoding sequence operably linked to an expression control sequence, and a genetic mutation that causes the host cell to have a greater likelihood of becoming a cancer cell than a cell not comprising the genetic mutation. Such a mutation can be, e.g., a mutation that deletes or inactivates a tumor suppressor gene, or a mutation that activates an oncogene. Examples of tumor suppressor genes include INK4a, P53, Rb, PTEN, LATS, APAF1, Caspase 8, APC, DPC4, KLF6, GSTP1, ELAC2/HPC2 and NKX3.1. Examples of oncogenes include K-RAS, H-RAS, N-RAS, EGFR, MDM2, TGF-β, RhoC, AKT family members, myc, β-catenin, PGDF, C-MET, PI3K-CA, CDK4, cyclin B1, cyclin D1, estrogen receptor gene, progesterone receptor gene, HER2 (also known as neu or ErbB2), ErbB1, ErbB3, ErbB4, TGFα, ras-GAP, Shc, Nck, Src, Yes, Fyn, Wnt, and Bcl2.

The invention also provides a genetically modified non-human mammal, e.g., a mouse, at least some of whose cells contain a genome that includes: (a) a recombinant GP153-encoding nucleic acid operably linked to an expression control sequence, and (b) a genetic mutation that causes the mammal to have a greater susceptibility to cancer than a mammal whose cells do not contain the genetic mutation. In preferred embodiments, the genetic mutation involves a tumor suppressor gene and renders the tumor suppressor gene non-functional. The genetically modified nonhuman mammal can be a conventional transgenic mammal, all of whose cells contain the recombinant GP153-encoding nucleic acid operably linked to an expression control sequence, and the genetic mutation that causes the mammal to have increased susceptibility to cancer. Alternatively, the mammal can be a chimeric mammal at least some of whose, but not all of whose, somatic cells contain the recombinant GP153-encoding nucleic acid operably linked to an expression control sequence, and the genetic mutation that causes the

mammal to have a increased susceptibility to cancer. In such a chimeric mammal, the percentage of somatic cells containing the recombinant GP153-encoding nucleic acid operably linked to an expression control sequence, and a genetic mutation that causes the mammal to have a greater susceptibility to cancer is between 5% and 95%. Preferably it is between 15% and 85%. In some embodiments of the invention, the GP153-encoding nucleic acid is operably linked to a tissue-specific expression system.

The invention also provides a genetically modified nonhuman mammal, wherein the genetic modification reduces or eliminates expression of one or both of the mammal's endogenous GP153 alleles. Such reduction or elimination of GP153 expression can be achieved, for example, when the genetic modification is addition of an RNAi expression construct targeting GP153 gene expression, or when the genetic modification is a knockout of one or both of the GP153 alleles. Such a genetic modification can reduce or eliminate GP153 expression in a tissue-specific manner. In some embodiments of the invention, the genetically modified mammal is chimeric with respect to the genetic modification.

The invention also provides a screening method for identifying a compound useful for treating a hyperproliferative condition such as cancer. The method includes: (a) identifying a biomarker whose level correlates with inhibition of GP153 activity; and (b) detecting a change in the level of the biomarker in the presence of a test compound relative to the level of the biomarker detected in the absence of the test compound.

The invention also provides a screening method for identifying a compound useful in treatment of a hyperproliferative condition such as cancer. The method includes: (a) providing an inhibitor of GP153 expression or activity; (b) identifying a negative control biomarker pattern formed by a plurality of biomarkers in a cancer cell wherein the cell is not contacted with the inhibitor of GP153 expression or activity; (c) identifying a positive control biomarker pattern formed by a plurality of biomarkers in the cancer cell wherein the cancer cell is contacted with the inhibitor of GP153 expression or activity; (d) identifying a test biomarker pattern formed by a plurality of biomarkers in the cancer cell wherein the cancer cell is contacted with a candidate compound but not contacted with the inhibitor of

GP153 expression or activity; and (e) comparing the negative control biomarker pattern, positive control biomarker pattern and test biomarker pattern, and detecting a greater similarity between the positive control biomarker pattern and the test biomarker pattern than between the negative control biomarker pattern and the test biomarker pattern.

The invention also provides methods of diagnosing an abnormal hyperproliferative condition, e.g., cancer, in a subject. These methods involve detecting the expression level of a GP153 gene or the activity level of a GP153 protein. An abnormally high level relative to control, e.g., at least about 50%, 100%, 150%, 200%, 250%, or 300% higher, indicates an abnormal hyperproliferative condition.

The invention also provides GP153 polypeptide antigens consisting essentially of amino acids 30-280; 30-320; 221-500; 236-488; 409-690; 500-704; 1-704; and 30-704 of SEQ ID NO:1. Any of these GP153 polypeptide antigens can be incorporated into a fusion protein, e.g., an Fc fusion or a GST fusion.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. In case of conflict, the present specification, including definitions, will control. All publications, patents and other references mentioned herein are incorporated by reference in their entirety.

Throughout this specification and claims, the word "comprise," or variations such as "comprises" or "comprising" is intended to include the stated integer or group of integers, but not to exclude any other integer or group of integers.

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are described below. The materials, methods and examples are illustrative only, and are not intended to be limiting. Other features and advantages of the invention will be apparent from the detailed description and from the claims.

Other features and advantages of the invention will be apparent from the following detailed description.

Detailed Description of the Invention

This invention is based in part on the discovery that gene designated GP153 is involved in hyperproliferative conditions such as cancer. Up-regulation of GP153 contributes to tumorigenesis and tumor maintenance in a mammal.

The GP153 protein, i.e., protein-tyrosine-kinase-7 or PTK7 or colon carcinoma kinase-4 or CCK4, belongs to a family of receptor tyrosine kinases (RTKs) that lack detectable catalytic tyrosine kinase activity. Nevertheless, this protein may have a role in signal transduction. It may interact with other protein kinases and mediate their activity or induce intracellular signaling by binding to extracellular ligands. An exemplary human GP153 protein has the following polypeptide sequence:

MGAARGSPAR PRRLPLLSVL LLPLLGGTQT AIVFIKQPSS QDALQGRRAL
LRCEVEAPGP VHVYWLLDGA PVQDTERRFA QGSSLSFAAV DRLQDSGTFQ
CVARDDVTGE EARSANASFN IKWIEAGPVV LKHPASEAEI QPQTQVTLRC
HIDGHPRPTY QWFRDGTPLS DGQSNHTVSS KERNLTLRPA GPEHSGLYSC
CAHSAFGQAC SSQNFTLSIA DESFARVVLA PQDVVVARYE EAMFHCQFSA
QPPPSLQWLF EDETPITNRS RPPHLRRATV FANGSLLLTQ VRPRNAGIYR
CIGQGQRGPP IILEATLHLA EIEDMPLFEP RVFTAGSEER VTCLPPKGLP
EPSVWWEHAG VRLPTHGRVY QKGHELVLAN IAESDAGVYT CHAANLAGQR
RQDVNITVAT VPSWLKKPQD SQLEEGKPGY LDCLTQATPK PTVVWYRNQM
LISEDSRFEV FKNGTLRINS VEVYDGTWYR CMSSTPAGSI EAQARVQVLE
KLKFTPPPQP QQCMEFDKEA TVPCSATGRE KPTIKWERAD GSSLPEWVTD
NAGTLHFARV TRDDAGNYTC IASNGPQGQI RAHVQLTVAV FITFKVEPER
TTVYQGHTAL LQCEAQGDPK PLIQWKGKDR ILDPTKLGPR MHIFQNGSLV
IHDVAPEDSG RYTCIAGNSC NIKHTEAPLY VVDKPVPEES EGPGSPPPYK
MIQTIGLSVG AAVAYIIAVL GLMFYCKKRC KAKRLQKQPE GEEPEMECLN
GGPLQNGQPS AEIQEEVALT SLGSGPAATN KRHSTSDKMH FPRSSLQPIT
TLGKSEFGEV FLAKAQGLEE GVAETLVLVK SLQSKDEQQQ LDFRRELEMF
GKLNHANVVR LLGLCREAEP HYMVLEYVDL GDLKQFLRIS KSKDEKLKSQ
PLSTKQKVAL CTQVALGMEH LSNNRFVHKD LAARNCLVSA QRQVKVSALG
LSKDVYNSEY YHFRQAWVPL RWMSPEAILE GDFSTKSDVW AFGVLMWEVF

THGEMPHGGQ ADDEVLADLQ AGKARLPQPE GCPSKLYRLM QRCWALSPKD
RPSFSEIASA LGDSTVDSKP (SEQ ID NO:1; GenBank No. NP_002812)

A coding sequence for this polypeptide is:

gcggcgcgcg gggactcgga ggtactgggc gcgcgcggct ccggctcggg
acgcctcggg acgcctcggg gtcgggctcc ggctgcggct gctgctgcgg
cgcccgcgct ccggtgcgct ccgcctcctg tgcccgccgc ggagcgcagt
ctgcgcgccc gccgtgcgcc ctcagctcct tttcctgagc ccgccgcgat
gggagctgcg cggggatccc cggccagacc ccgccggttg cctctgctca
gcgtcctgct gctgccgctg ctgggcggta cccagacagc cattgtcttc
atcaagcagc cgtcctccca ggatgcactg caggggcgcc gggcgctgct
tcgctgtgag gttgaggctc cgggcccggt acatgtgtac tggctgctcg
atggggcccc tgtccaggac acggagcggc gtttcgccca gggcagcagc
ctgagctttg cagctgtgga ccggctgcag gactctggca ccttccagtg
tgtggctcgg gatgatgtca ctggagaaga agcccgcagt gccaacgcct
ccttcaacat caaatggatt gaggcaggtc ctgtggtcct gaagcatcca
gcctcggaag ctgagatcca gccacagacc caggtcacac ttcgttgcca
cattgatggg caccctcggc ccacctacca atggttccga gatgggaccc
ccctttctga tggtcagagc aaccacacag tcagcagcaa ggagcggaac
ctgacgctcc ggccagctgg tcctgagcat agtgggctgt attcctgctg
cgcccacagt gcttttggcc aggcttgcag cagccagaac ttcaccttga
gcattgctga tgaaagcttt gccagggtgg tgctggcacc ccaggacgtg
gtagtagcga ggtatgagga ggccatgttc cattgccagt tctcagccca
gccacccccg agcctgcagt ggctctttga ggatgagact cccatcacta
accgcagtcg ccccccacac ctccgcagag ccacagtgtt tgccaacggg
tctctgctgc tgacccaggt ccggccacgc aatgcaggga tctaccgctg
cattggccag gggcagaggg gcccacccat catcctggaa gccacacttc
acctagcaga gattgaagac atgccgctat ttgagccacg ggtgtttaca
gctggcagcg aggagcgtgt gacctgcctt ccccccaagg gtctgccaga
gcccagcgtg tggtgggagc acgcgggagt ccggctgccc acccatggca
gggtctacca gaagggccac gagctggtgt tggccaatat tgctgaaagt
gatgctggtg tctacacctg ccacgcggcc aacctggctg gtcagcggag
acaggatgtc aacatcactg tggccactgt gccctcctgg ctgaagaagc

cccaagacag ccagctggag gagggcaaac ccggctactt ggattgcctg
acccaggcca caccaaaacc tacagttgtc tggtacagaa accagatgct
catctcagag gactcacggt tcgaggtctt caagaatggg accttgcgca
tcaacagcgt ggaggtgtat gatgggacat ggtaccgttg tatgagcagc
accccagccg gcagcatcga ggcgcaagcc cgtgtccaag tgctggaaaa
gctcaagttc acaccaccac cccagccaca gcagtgcatg gagtttgaca
aggaggccac ggtgccctgt tcagccacag gccgagagaa gcccactatt
aagtgggaac gggcagatgg gagcagcctc ccagagtggg tgacagacaa
cgctgggacc ctgcattttg cccgggtgac tcgagatgac gctggcaact
acacttgcat tgcctccaac gggccgcagg gccagattcg tgcccatgtc
cagctcactg tggcagtttt tatcaccttc aaagtggaac cagagcgtac
gactgtgtac cagggccaca cagccctact gcagtgcgag gcccaggggg
accccaagcc gctgattcag tggaaaggca aggaccgcat cctggacccc
accaagctgg gacccaggat gcacatcttc cagaatggct ccctggtgat
ccatgacgtg gcccctgagg actcaggccg ctacacctgc attgcaggca
acagctgcaa catcaagcac acggaggccc ccctctatgt cgtggacaag
cctgtgccgg aggagtcgga gggccctggc agccctcccc cctacaagat
gatccagacc attgggttgt cggtgggtgc cgctgtggcc tacatcattg
ccgtgctggg cctcatgttc tactgcaaga agcgctgcaa agccaagcgg
ctgcagaagc agcccgaggg cgaggagcca gagatggaat gcctcaacgg
tgggcctttg cagaacgggc agccctcagc agagatccaa gaagaagtgg
ccttgaccag cttgggctcc ggccccgcgg ccaccaacaa acgccacagc
acaagtgata agatgcactt cccacggtct agcctgcagc ccatcaccac
gctggggaag agtgagtttg gggaggtgtt cctggcaaag gctcagggct
tggaggaggg agtggcagag accctggtac ttgtgaagag cctgcagagc
aaggatgagc agcagcagct ggacttccgg agggagttgg agatgtttgg
gaagctgaac cacgccaacg tggtgcggct cctggggctg tgccgggagg
ctgagcccca ctacatggtg ctggaatatg tggatctggg agacctcaag
cagttcctga ggatttccaa gagcaaggat gaaaaattga agtcacagcc
cctcagcacc aagcagaagg tggccctatg cacccaggta gccctgggca
tggagcacct gtccaacaac cgctttgtgc ataaggactt ggctgcgcgt
aactgcctgg tcagtgccca gagacaagtg aaggtgtctg ccctgggcct
cagcaaggat gtgtacaaca gtgagtacta ccacttccgc caggcctggg

tgccgctgcg ctggatgtcc cccgaggcca tcctggaggg tgacttctct
accaagtctg atgtctgggc cttcggtgtg ctgatgtggg aagtgtttac
acatggagag atgccccatg gtgggcaggc agatgatgaa gtactggcag
atttgcaggc tgggaaggct agacttcctc agcccgaggg ctgcccttcc
aaactctatc ggctgatgca gcgctgctgg gccctcagcc ccaaggaccg
gccctccttc agtgagattg ccagcgccct gggagacagc accgtggaca
gcaagccgtg aggagggagc ccgctcagga tggcctgggc aggggaggac
atctctagag ggaagctcac agcatgatgg gcaagatccc tgtcctcctg
ggccctgagg cccctgccct agtgcaacag gcattgctga ggtctgagca
gggcctggcc tttcctcctc ttcctcaccc tcatcctttg ggaggctgac
ttggacccaa actgggcgac tagggctttg agctgggcag ttttccctgc
cacctcttcc tctatcaggg acagtgtggg tgccacaggt aaccccaatt
tctggccttc aacttctccc cttgaccggg tccaactctg ccactcatct
gccaactttg cctggggagg gctaggcttg ggatgagctg ggtttgtggg
gagttcctta atattctcaa gttctgggca cacagggtta atgagtctct
tggcccactg gtcccacttg ggggtctaga ccaggattat agaggacaca
gcaagtgagt cctccccact ctgggcttgt gcacactgac ccagacccac
gtcttcccca cccttctctc ctttcctcat cctaagtgcc tggcagatga
aggagttttc aggagctttt gacactatat aaaccgccct ttttgtatgc
accacgggcg gcttttatat gtaattgcag cgtggggtgg gtgggcatgg
gaggtagggg tgggccctgg agatgaggag ggtgggccat ccttacccca
cacttttatt gttgtcgttt tttgtttgtt ttgtttttt gtttttgttt
ttgtttttac actcgctgct ctcaataaat aagccttttt tacaacctg
(SEQ ID NO:2; GenBank Accession No. NM_002821)

The open reading frame of the above sequence is nucleotides 199-3411. Other human GP153 sequences include transcript variants encoding different isoforms of GP153. Other human GP153 polypeptide sequences include different isoforms of GP153 such as GenBank accession numbers NP_690619, NP_690620, NP_690621, and NP_690622. Other human GP153 coding sequences include GenBank accession numbers NM_152880.2, NM_152881.2, NM_152882.2, NM_152883.1, BC046109.2, AK124108.1, NM_002821.3, AL157486.1, U33635.1, U40271.2, AK055648.1, BC014626.1, AF531872.1, AF531871.1,

AF531870.1, AF531869.1, AF531868.1, and BC002377.1. GP153 orthologs in other animal species have also been identified. They include GenBank accession numbers BE233531.1 (*S.scrofa*), XM_217346.2 (*R.norvegicus*), NM_175168.2 (*M.musculus*), and BF606652.1 (*B.taurus*).

The GP153 gene is expressed in a variety of tissues, including adipose tissue, eye (including retina and lens), fetus, gastrointestinal tract (including colon), genitourinary organs (e.g., prostate, testis, and ovaries), heart, kidney, lung, breast, stomach, nervous systems (e.g., brain), pancreas, placenta, spleen, thymus, and uterus. The GP153 gene is also expressed in a broad spectrum of cancer tissue types (e.g., adenocarcinoma, colon tumor, prostate tumor, squamous cell carcinoma, rhabdomyosarcoma, neuroblastoma, mucoepidermoid carcinoma, and retinoblastoma) and their derivative cancer cell lines (e.g., cancer cell lines derived from neuroblastoma and adenocarcinoma). GP153 is also expressed at elevated levels in brain tumors, colon cancer and lung cancer.

GP153 antagonists are useful for inhibiting, treating or preventing the development of cancers such as cancers found in skin (e.g., melanoma), lung, prostate, breast, colorectal, liver, pancreatic, brain, testicular, ovarian, uterine, cervical, kidney, thyroid, bladder, esophageal, and hematological tissues.

Inhibition of GP153 function inhibits the growth of different human tumor cell lines in a soft agar colony formation assay (Example 3, below). This demonstrates a role for GP153 in the regulation of tumor cell growth.

GP153 may form heterodimers with other active RTKs, which themselves can be targeted for drug discovery efforts. Potential partners of GP153 can be found by searching for RTKs that display the same expression pattern as GP153. Alternatively, partners of GP153 can be found by using proteomic or yeast two-hybrid approaches. For example, in one approach, GP153 can be crosslinked to associated proteins using chemical crosslinking agents, and the crosslinked GP153-protein complex can be isolated using antibodies specific to GP153. The GP153 associated proteins or their fragments can be analyzed using mass spectrometry to identify the GP153 associated, catalytically active RTK.

In another approach, the extracellular or intracellular domain of GP153 can be used as a bait in a yeast two-hybrid experiment to identify RTKs that form heterodimers with GP153. This approach can also be used to identify proteins that interact with the intracellular domain of GP153 and are therefore likely to be downstream effectors of GP153, and may be additional drug targets in the GP153 pathway. In addition, this approach can be used to identify proteins that interact with the extracellular domain of GP153, including ligand(s) for GP153. Antibodies that bind to the activating ligand of GP153, and prevent its binding to the extracellular domain of GP153, may provide an additional therapeutic intervention point in the GP153 pathway.

GP153-Related Nucleic Acids

The nucleic acid sequences specifically provided herein are sequences of deoxyribonucleotides. However, the given sequences are to be interpreted as would be appropriate to the polynucleotide composition. For example, if the isolated nucleic acid is RNA, the given sequence intends ribonucleotides, with uridine substituted for thymidine. In some embodiments, differences from naturally occurring nucleic acids, e.g., non-native bases, altered internucleoside linkages, and post-synthesis modification, can be present throughout the length of the GP153 nucleic acid or can be usefully localized to discrete portions thereof. For example, a chimeric nucleic acid can be synthesized with discrete DNA and RNA domains and demonstrated utility for targeted gene repair. See, e.g., U.S. Pat. Nos. 5,760,012 and 5,731,181.

Polymorphisms such as single nucleotide polymorphisms (SNPs) occur frequently in eukaryotic genomes. Additionally, small deletions and insertions, rather than SNPs, are not uncommon in the general population, and often do not alter the function of the protein. Accordingly, this invention provides not only isolated nucleic acids identical in sequence to those described with particularity herein, but also isolated nucleic acids that are allelic variants of those particularly described nucleic acid sequences. In some embodiments, such sequence variations result from human intervention, e.g., by random or directed mutagenesis.

Nucleic Acids Encoding GP153 Protein or Portions Thereof

The invention provides isolated nucleic acid molecules that encode the entirety or part (e.g., at least five, seven, or nine contiguous amino acid residues) of the GP153 protein, including allelic variants of this protein.

These nucleic acids can be used, for example, to express the GP153 protein or specific portions of the protein, either alone or as elements of a fusion protein, e.g., to express epitopic or immunogenic fragments of the GP153 protein. For example, such nucleic acids are used to produce non-human mammals of the invention. These nucleic acids also can be used as probes to hybridize to GP153 nucleic acids and related nucleic acid sequences.

This invention also relates to nucleic acids comprising sequences coding for polypeptides containing conservative amino acid substitutions or moderately conservative amino acid substitutions from those polypeptides described with particularity herein. These amino acid substitutions can be due to, e.g., allelic variations, naturally occurring mutations, or man-made mutations.

Cross-Hybridizing Nucleic Acids

This invention also relates to isolated polynucleotides that hybridize to one or more of the above-described GP153 nucleic acids. These cross-hybridizing nucleic acids can be used, e.g., as hybridization probes, primers, and/or for expression of proteins that are related to GP153 as isoforms and homologs, e.g., paralogs, and orthologs. In some such embodiments, the invention relates to an isolated nucleic acid comprising a sequence that hybridizes under high stringency conditions to a probe comprising a fragment of SEQ ID NO: 2 having at least 15, 16, 18, 20, 24, or 25 nucleotides. As used herein, "high stringency conditions" are defined for solution phase hybridization as aqueous hybridization (i.e., free of formamide) in 6X SSC (where 20X SSC contains 3.0 M NaCl and 0.3 M sodium citrate), 1% SDS at 65°C for 8-12 hours, followed by two washes in 0.2X SSC, 0.1% SDS at 65°C for 20 minutes. It will be appreciated by the skilled worker that hybridization at 65°C will occur at different rates depending on a number of factors including the length and percent identity of the sequences which are hybridizing.

The hybridizing portion of a reference nucleic acid (i.e., target nucleic acid) is typically at least 15 nucleotides in length, and often at least 17 , 20, 25, 30, 35, 40 or 50 nucleotides in length. Cross-hybridizing nucleic acids that hybridize to a larger portion of the reference nucleic acid – for example, to a portion of at least 50, 100, 150, 200, 250, 300, 350, 400, 450, 500 or more nucleotides, up to and including the entire length of the reference nucleic acid, are also useful.

Nucleic Acid Fragments

Fragments of the above-described nucleic acids also relate to this invention. They can be used as region-specific probes, as amplification primers, regulatory sequences to direct expression of a gene, and/or to direct expression of a GP153 polypeptide fragment, e.g., immunogenic fragment.

The nucleic acid probes may comprise a detectable label, e.g., a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as a part of a diagnostic kit for identifying cells or tissues that (i) incorrectly express a GP153 protein, e.g., aberrant splicing, or abnormal mRNA levels, or (ii) harbor a mutation in the GP153 gene, such as a deletion, an insertion, or a point mutation. Such diagnostic kits preferably include labeled reagents and instructional inserts for their use.

The nucleic acid primers can be used in PCR, primer extension and the like. They can be, e.g., at least 6 nucleotides (e.g., at least 7, 8, 9, or 10) in length. The primers can hybridize to an exon sequence of a GP153 gene, e.g., for amplification of a GP153 mRNA or cDNA. Alternatively, the primers can hybridize to an intron sequence or an upstream or downstream regulatory sequence of a GP153 gene, to utilize non-transcribed, e.g., regulatory portions of the genomic structure of a GP153 gene. The nucleic acid primers also can be used, e.g., to prime single base extension (SBE) for SNP detection (see, e.g., U.S. Pat. No. 6,004,744). Isothermal amplification approaches, such as rolling circle amplification, are also now well-described. See, e.g., Schweitzer et al., Curr. Opin. Biotechnol. 12(1):21-7 (2001); U.S. Patent Nos. 5,854,033 and 5,714,320 and PCT international patent publications WO 97/19193 and WO 00/15779. Rolling circle amplification can be

combined with other techniques to facilitate SNP detection. See, e.g., Lizardi et al., Nature Genet. 19:225-32 (1998).

Nucleic acid fragments that encode 5 or more contiguous amino acids, e.g., fragments of 15, 18, 21, 24, or 27 nucleotides or more, are useful in directing the synthesis of peptides that have utility in mapping the epitopes of the GP153 protein. See, e.g., Geysen et al., Proc. Natl. Acad. Sci. USA 81:3998-4002 (1984); and U.S. Pat. Nos. 4,708,871 and 5,595,915. Such nucleic acid fragments are also useful in directing the synthesis of peptides that have utility as immunogens. See, e.g., Lerner, Nature 299:592-596 (1982); Shinnick et al., Annu. Rev. Microbiol. 37:425-46 (1983); Sutcliffe et al., Science 219:660-6 (1983). Larger fragments containing at least 25, 30, 35, 40, 45, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500 or more nucleotides are also useful and sometimes preferred.

Single Exon Probes

The invention also relates to single exon probes having portions of no more than one exon of the GP153 gene. Such single exon probes have particular utility in identifying and characterizing splice variants. In particular, these probes are useful for identifying and discriminating the expression of distinct isoforms of GP153.

Antisense Reagents

Some embodiments of the invention relate to antisense polynucleotides that specifically hybridize to GP153 sense polynucleotides. The antisense nucleic acid molecule can be complementary to the entire coding or non-coding region of GP153, but more often is antisense to only a portion of the coding or non-coding region of GP153 mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of GP153 mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length.

The antisense nucleic acids of this invention, for example, may form a stable duplex with its target sequence, or, in the case of an antisense nucleic acid molecule that binds to DNA duplexes, through specific interactions in the major groove of the double helix. In other embodiments, the antisense nucleic acid

molecule of the invention is an α-anomeric nucleic acid molecule. An α-anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gaultier et al. Nucl. Acids Res 15: 6625-6641 (1987)).

An antisense target sequence is a nucleotide sequence specific to GP153, and can be designed through use of a publicly available sequence database, and/or through use of commercially available sequence comparison programs. Antisense nucleic acids of the invention can then be constructed using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be produced biologically using an expression vector into which a nucleic acid has been inserted in an antisense orientation, i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest.

Alternatively, the antisense nucleic acid can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecule or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids. For example, phosphorothioate derivatives and acridine substituted nucleotides can be used. Phosphorothioate and methylphosphonate antisense oligonucleotides are useful in practicing the invention. The antisense nucleic acid molecule can also comprise a 2'-O-methylribonucleotide (Inoue et al., Nucl. Acids Res. 15: 6131-6148 (1987)) or a chimeric RNA -DNA analogue (Inoue et al., FEBS Lett. 215: 327-330 (1987)). The antisense oligonucleotides may be further modified by adding poly-L-lysine, transferrin, polylysine, or cholesterol moieties at their 5' end.

The antisense molecules will be tested for undesired non-specific effects such as the induction of ds-RNA stress response genes in the interferon pathway. Only those molecules that do not induce a significant non-specific response will be subsequently used.

Antisense molecules can be administered to a mammal or generated *in situ* via an expression vector, such that they bind to cellular RNA and/or genomic DNA encoding a GP153 protein, thereby inhibiting GP153 expression. Suppression of GP153 expression at either the transcriptional or translational level is useful to

treat certain cancer conditions in patients or to generate cellular or animal models for cancer characterized by aberrant GP153 expression. An antisense molecule can be administered by direct injection at a tissue site of a subject. Alternatively, an antisense molecule can be designed to target selected cells, e.g., cancer cells overexpressing GP153, and then administered systemically.

Ribozymes and Catalytic Nucleic Acids

In some embodiments, an antisense nucleic acid of the invention is part of a GP153-specific ribozyme (or, as modified, a "nucleozyme"). Ribozymes are catalytic RNA molecules with ribonuclease activity capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes such as hammerhead, hairpin, and Group I intron ribozymes can cleave GP153 mRNA transcripts catalytically, thereby inhibiting translation of GP153 mRNA. A ribozyme having specificity for a GP153-encoding nucleic acid can be designed based upon the nucleotide sequence of a GP153 polynucleotide disclosed herein (SEQ ID NO: 2). See, e.g., U.S. Patent Nos. 5,116,742; 5,334,711; 5,652,094; and 6,204,027. For example, a derivative of a Tetrahymena L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a GP153-encoding mRNA. See, e.g., Cech et al. U.S. Pat. Nos. 4,987,071 and 5,116,742. Alternatively, GP153 mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, e.g., Bartel et al., Science 261:1411-1418 (1993).

In some embodiments, the ribozymes and other antisense reagents of this invention include appended groups such as peptides. This is useful for targeting host cell receptors, facilitating transport across the cell membrane (Letsinger et al., Proc. Natl. Acad. Sci. USA 86:6553-6556 (1989); Lemaitre et al., Proc. Natl. Acad. Sci. USA 84:648-652 (1987); WO 88/09810), or facilitating transport across the blood-brain barrier (WO 89/10134).

Expression of the GP153 gene can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the GP153, e.g., the GP153 promoter and/or enhancers, to form triple helical structures that prevent

transcription of the GP153 gene in target cells. See generally, Helene, Anticancer Drug Des. 6: 569-84 (1991); Helene et al., Ann. N.Y. Acad. Sci. 660:27-36 (1992); and Maher, Bioassays 14: 807-15 (1992).

Peptide Nucleic Acids (PNA)

Some preferred oligonucleotide mimetics, especially those useful for *in vivo* administration, are peptide nucleic acids (PNA). See, e.g., Hyrup et al., Bioorg. Med. Chem. Lett. 4:5-23 (1996). In PNA compounds, the phosphodiester backbone of the nucleic acid is replaced with an amide-containing backbone, in particular by repeating N-(2-aminoethyl) glycine units linked by amide bonds. Nucleobases are bound directly or indirectly to aza-nitrogen atoms of the amide portion of the backbone, typically by methylene carbonyl linkages. The synthesis of PNA oligomers can be performed using conventional solid phase peptide synthesis as described in Hyrup et al., *supra*; and Perry-O'Keefe et al., Proc. Natl. Acad. Sci. USA 93:14670-675 (1996).

GP153-based PNAs can be used in therapeutic and diagnostic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, e.g., inducing transcription or translation arrest or inhibiting replication. GP153-based PNAs also can be used in the analysis of single base pair mutations in a gene. This can be done by PNA directed PCR clamping; as artificial restriction enzymes when used in combination with other enzymes, e.g., S1 nucleases, or as probes or primers for DNA sequence and hybridization (Hyrup et al., *supra*; Perry-O'Keefe, *supra*).

In other embodiments, PNAs of GP153 are modified, e.g., to enhance their stability or cellular uptake, by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery known in the art. For example, PNA-DNA chimeras of GP153 can be generated that may combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes, e.g., RNase H and DNA polymerases, to interact with the DNA portion while the PNA portion would provide high binding affinity and specificity. PNA-DNA chimeras can be linked using linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup et al., *supra*). The

synthesis of PNA-DNA chimeras can be performed as described in Hyrup, *supra* and Finn et al., Nucl. Acids Res. 24:3357-63 (1996).

RNA Interference

The invention provides RNA interference (RNAi) for use in silencing the expression of the GP153 gene. RNAi is a sequence-specific posttranscriptional gene silencing mechanism triggered by double-stranded RNA (dsRNA) and causes degradation of mRNAs homologous in sequence to the dsRNA. The mediators of the degradation are 21- to 23-nucleotide small interfering RNAs (siRNAs) generated by ribonuclease III cleavage from the longer dsRNAs. Molecules of siRNA typically have 2- to 3-nucleotide 3' overhanging ends resembling the RNAse III processing products of long dsRNAs that normally initiate RNAi. When introduced into a cell, they assemble with an endonuclease complex (RNA-induced silencing complex), which then guides target mRNA cleavage. As a consequence of degradation of the targeted mRNA, cells with a specific phenotype of the suppression of the corresponding protein product are obtained (e.g., reduction of tumor size, metastasis, angiogenesis, and growth rates). The small size of siRNAs, compared with traditional antisense molecules, prevents activation of the dsRNA-inducible interferon system present in mammalian cells. This helps avoid the nonspecific phenotypes normally produced by dsRNA larger than 30 base pairs in somatic cells. For review, see, e.g., Elbashir et al., Methods 26:199-213 (2002); McManus et al., Nature Rev. 3:737-747 (2002); Hannon, Nature 418:244-251 (2002); Tuschl, Nature Biotech. 20:446-448 (2002); and Tuschl U.S. Pat. Appln US2002/0086356.

Small interfering RNA oligonucleotides can be designed by using any one of a number of software programs, e.g., the OligoEngine™ siRNA design tool available at www.oligoengine.com; and RNA Oligo Retriever design tool available from Cold Spring Harbor Laboratory at www.cshl.org/public/SCIENCE/hannon.html (see Paddison et al., 2002, Genes & Dev.16: 948-958). Alternatively, suitable siRNAs can be purchased commercially from vendors such as Dharmacon RNA Technologies (LaFayette, CO). Preferred siRNAs of this invention range about 19-29 base pairs in length for the double-stranded portion. In some embodiments, the siRNAs are hairpin RNAs having an

about 19-29 bp stem and an about 4-34 nucleotide loop. Preferred siRNAs are highly specific for a GP153 target region and may comprise any 19-29 bp fragment of a GP153 mRNA that has at least 1 (e.g., at least 2 or 3) bp mismatch with a nonGP153 -related sequence. In some embodiments, the preferred siRNAs do not bind to RNAs having more than 3 mismatches with the target region.

The target sequences of exemplary siRNAs for GP153 are:

5'-gggagctgcgcggggatccccggccagac-3'(SEQ ID NO:3);

5'-ctgggagacagcaccgtggacagcaagcc-3' (SEQ ID NO:4);

5'-ccgagagaagcccactattaagtgggaac-3' (SEQ ID NO:5); and

5'-acgtggtagtagcgaggtatgaggaggcc-3' (SEQ ID NO:6).

These sequences correspond to nucleotides 201-29, 3379-3407, 1782-1810, and 896-924 respectively of SEQ ID NO:2.

Exemplary 19-mer target sequences for GP153 are:

5'-cacttcgttgccacattga-3' (SEQ ID NO:7);

5'–cttcgttgccacattgatg-3' (SEQ ID NO: 8);

5'–gccagaacttcaccttgag-3' (SEQ ID NO: 9); and

5'-cagcacaagtgataagatg-3' (SEQ ID NO:10).

These sequences correspond to nucleotides 638-56, 640-58, 833-51, and 2547-65 respectively of SEQ ID NO:2. SiRNAs that target an RNA region having 10 or more nucleotide overlaps with an aforementioned exemplary target region are also useful.

Intracellular transcription of siRNAs can be achieved by cloning the siRNA templates into RNA polymerase III (Pol III) transcription units, which normally encode the small nuclear RNA U6 or the human RNAse P RNA H1. Two approaches can be used for expressing siRNA: (1) sense and antisense strands constituting the siRNA duplex are transcribed by individual promoters; or (2) siRNAs are expressed as fold-back stem-loop structures that give rise to siRNAs

after intracellular processing. Inducible promoters can also be used to drive the expression of the siRNA.

To target the regions of a GP153 mRNA corresponding to SEQ ID NOs:3, 4, 5, and 6, the following oligonucleotides can be used with a primer specific to the U6 small RNA promoter to form double-stranded DNA in a polymerase chain reaction, using a vector containing this U6 promoter as a template. The PCR product can be ligated into a vector. Expression of the insert will lead to expression of a short hairpin RNA (shRNA). The shRNA will have inhibitory effects on GP153 expression.

1) Oligonucleotide to target SEQ ID NO:3

ggaattcaaaaaatccggccgaggatccccacgcagctcccccaa**gggagctgcg cggggatccccggccagac**tagtatatgtgctgccgaagc (SEQ ID NO:11; SEQ ID NO:3 in boldface)

2) Oligonucleotide to target SEQ ID NO:4

ggaattcaaaaaggcctgctgcccacgatgctgcctcccagccaa**ctgggagaca gcaccgtggacagcaagcc**tagtatatgtgctgccgaagc (SEQ ID NO:12; SEQ ID NO:4 in boldface)

3) Oligonucleotide to target SEQ ID NO:5

ggaattcaaaaagttcccactcaatagcgggctcctctcagccaa**ccgagagaag cccactattaagtgggaac**tagtatatgtgctgccgaagc (SEQ ID NO:13; SEQ ID NO:5 in boldface)

4) Oligonucleotide to target SEQ ID NO:6

ggaattcaaaaaagcctcctcatacctcgccaccaccacatccaa**acgtggtagt agcgaggtatgaggaggcc**tagtatatgtgctgccgaagc (SEQ ID NO:14; SEQ ID NO:6 in boldface)

An siRNA oligonucleotide or its coding sequence can be delivered into a target cell via a variety of methods, including but not limited to, liposome fusion (transposomes), routine nucleic acid transfection methods such as electroporation, calcium phosphate precipitation, and microinjection, and infection by viral vectors.

Exemplary Uses of GP153 Nucleic Acids

The above-described nucleic acids can be used as hybridization probes to characterize GP153 nucleic acids in both genomic and transcript-derived nucleic acid samples. For example, the probes can be used to detect gross alterations in the GP153 genomic locus, such as deletions, insertions, translocations, and duplications of the GP153 genomic locus. Methods of detection include fluorescence *in situ* hybridization (FISH) to chromosome spreads, comparative genomic hybridization (CGH), array CGH (e.g., on microarrays containing GP153 -coding sequences or BAC comprising GP153 -coding sequences), and spectral karyotyping (SKY). See, e.g., Andreeff et al. (eds.), Introduction to Fluorescence In Situ Hybridization: Principles and Clinical Applications, John Wiley & Sons (1999) (ISBN: 0471013455). The probes can also be used to assess smaller genomic alterations using, e.g., Southern blot detection of restriction fragment length polymorphisms. The nucleic acid probes can be also used to isolate genomic clones that include the nucleic acids of the present invention, which thereafter can be restriction mapped and sequenced to identify deletions, insertions, amplifications, translocations, and substitutions (e.g., SNPs) at the sequence level. The nucleic acid probes can also be used to isolate GP153 nucleic acids from cDNA libraries, permitting sequence level characterization of GP153 RNA messages, including identification of deletions, insertions, truncations (including deletions, insertions, and truncations of exons in alternatively spliced forms) and single nucleotide polymorphisms. Some nucleic acids of this invention can also be used as amplification primers for PCR (e.g., real time PCR) to detect the above-described genomic alterations. Such genomic alterations of the GP153 gene often play a role in tumor genesis, maintenance and development, and thus can be used as markers for diagnosis and prognosis of GP153-mediated cancers.

The nucleic acid probes can be used to measure the representation of GP153 clones in a cDNA library, used as primers for quantitative real time PCR, or otherwise used to measure expression level of the GP153 gene. Measurement of GP153 expression has particular utility in diagnostic assays for cancer-related conditions associated with abnormal GP153 expression. Moreover, differences in the expression levels of the gene before and after a cancer event (e.g., cancer

genesis, maintenance, regression, and metastasis) are useful in determining the effect of a candidate cancer drug, identifying cancer types, designing diagnostics and prognostics, and predicting likely outcome of a cancer therapy.

The nucleic acids of this invention can also be used to introduce mutations (e.g., null mutations, dominant negative mutations, dominant acting mutations) into a GP153 locus of an animal via homologous recombination. Such animals (e.g., knock out mice) are useful in delineating the role of GP153 in tumor genesis and development and in facilitating cancer drug development.

Where the genomic region includes transcription regulatory elements, homologous recombination can be used to replace the endogenous regulatory elements with heterologous regulatory elements, i.e., elements not natively associated with the gene in the same manner. This can alter the expression of GP153, both for production of GP153 protein, and for gene therapy. See, e.g., U.S. Pat. Nos. 5,981,214, 6,048,524; and 5,272,071.

Fragments of the polynucleotides of the present invention smaller than those typically used for homologous recombination can also be used for targeted gene correction or alteration, possibly by cellular mechanisms different from those engaged during homologous recombination. See, e.g., U.S. Pat. Nos. 5,945,339, 5,888,983, 5,871,984, 5,795,972, 5,780,296, 5,760,012, 5,756,325, 5,731,181; and Culver et al., "Correction of chromosomal point mutations in human cells with bifunctional oligonucleotides," Nature Biotechnol. 17(10):989-93 (1999); Gamper et al., Nucl. Acids Res. 28(21):4332-9 (2000).

Vectors and Host Cells

General Consideration

This invention relates to nucleic acid constructs containing one or more of the isolated nucleic acid molecules of the invention. The vectors can be used to propagate the new nucleic acid molecules in host cells, to shuttle the molecules between host cells derived from disparate organisms, to insert the molecules into host genomes, to express sense or antisense RNA transcripts or interfering RNAs, and/or to express GP153 polypeptides. Typically, the vectors are derived from virus, plasmid, prokaryotic or eukaryotic chromosomal elements, or some

combination thereof, and may optionally include at least one origin of replication, at least one site for insertion of heterologous nucleic acid, and at least one selectable marker.

This invention relates to host cells, which can be either prokaryotic (bacteria) or eukaryotic, e.g., yeast, insect, plant and animal cells. A host cell strain may be chosen for its ability to process the expressed protein in the desired fashion. Such post-translational modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, hydroxylation, sulfation, lipidation, and acylation. Some embodiments of the invention may involve GP153 proteins with such post-translational modifications.

Exemplary prokaryotic host cells are *E. coli, Caulobacter crescentus, Streptomyces species,* and *Salmonella typhimurium* cells. Vectors useable in these cells include, without limitation, those related to pBR322 and the pUC plasmids.

Exemplary yeast host cells are *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Pichia pastoris,* and *Pichia methanolica.* Vectors useable in these host cells are integrative YIp vectors, replicating episomal YEp vectors containing centromere sequences CEN and autonomously replicating sequences ARS.

Insect cells are often chosen for high efficiency protein expression. Exemplary insect host cells are those from *Spodoptera frugiperda* (e.g., Sf9 and Sf21 cell lines, and EXPRESSF™ cells (Protein Sciences Corp., Meriden, CT, USA)), *Drosophila* S2 cells, and *Trichoplusia ni* HIGH FIVE® Cells (Invitrogen, Carlsbad, CA, USA). Where the host cells are *Spodoptera frugiperda* cells, the vector replicative strategy is typically based upon the baculovirus life cycle.

Exemplary mammalian host cells are COS1 and COS7 cells, NS0 cells, Chinese hamster ovary (CHO) cells, NIH 3T3 cells, 293 cells, HEPG2 cells, HeLa cells, L cells, MDCK, HEK293, WI38, murine ES cell lines (e.g., from strains 129/SV, C57/BL6, DBA-1, 129/SVJ), K562, Jurkat cells, BW5147 and any other commercially available human cancer cell lines. Cells with K-*ras*^{G13D}, such as human colon cancer cell lines DLD-1 and HCT-116, and revertants thereof having a null mutation in the activated K-*ras* gene, can also be used. Other useful

mammalian cell lines are well known and readily available from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and the National Institute of General medical Sciences (NIGMS) Human Genetic Cell Repository at the Coriell Cell Repositories (Camden, NJ, USA). As used herein, mammalian host cells also include those in the body of a subject (e.g., a human patient or an animal).

Vectors intended for autonomous extrachromosomal replication in mammalian cells will typically include a viral origin, such as the SV40 origin (for replication in cell lines expressing the large T-antigen, such as COS1 and COS7 cells), the papillomavirus origin, or the EBV origin for long term episomal replication (for use in, e.g., 293-EBNA cells, which constitutively express the EBV EBNA-1 gene product and adenovirus E1A). Vectors intended for integration, and thus replication as part of the mammalian chromosome, can, but need not, include an origin of replication functional in mammalian cells, such as the SV40 origin. Useful vectors also include vectors based lentiviruses, adenovirus, adeno-associated virus, vaccinia virus, parvoviruses, herpesviruses, poxviruses, Semliki Forest viruses, and retroviruses.

Plant cells can also be used for expression, with the vector replicon typically derived from a plant virus (e.g., cauliflower mosaic virus (CaMV); tobacco mosaic virus (TMV)) and selectable markers chosen for suitability in plants.

The invention relates to artificial chromosomes, e.g., bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), mammalian artificial chromosomes (MACs), and human artificial chromosomes (HACs), that contain the GP153 nucleic acid of interest.

Vectors often include elements that permit *in vitro* transcription of RNA from the inserted heterologous nucleic acid. Such vectors typically include a phage promoter, such as that from T7, T3, or SP6, flanking the nucleic acid insert. Often two different such promoters flank the inserted nucleic acid, permitting separate *in vitro* production of both sense and antisense strands.

Transcription Regulators for Expression Vectors

Expression vectors often include a variety of other genetic elements operatively linked to the protein-encoding heterologous nucleic acid insert. Examples of other genetic elements are promoters and enhancer elements, elements that facilitate RNA processing, e.g., transcription termination, splicing signals and/or polyadenylation signals, and elements that facilitate translation, e.g., ribosomal consensus sequences. Examples of other transcription control sequences include operators and silencers. Use of such expression control elements, including those that confer constitutive or inducible expression, and developmental or tissue-regulated expression, is within ordinary skill in the art.

Useful constitutive promoters include, without limitation, a CMV promoter, EF1α, retroviral LTRs, and SV40 early region. Inducible promoters useful in this invention include, without limitation, a tetracycline-inducible promoter, a metallothionine promoter, the IPTG/lacI promoter system, the ecdysone promoter system, and the "lox stop lox" system for irreversibly deleting inhibitory sequences for translation or transcription. In some embodiments, a GP153 gene is placed between lox sites. Upon expression of the cre enzyme, the GP153 gene is deleted from the genome so that the GP153 activity is permanently eliminated.

Instead of inducible promoters, the activity of a GP153 protein also can be inducibly switched on or off by fusing the GP153 protein to, e.g., an estrogen receptor polypeptide sequence, where administration of estrogen or an estrogen analog (e.g., hydroxytamoxifen) will allow the correct folding of the GP153 polypeptide into a functional protein.

Tissue-specific promoters that can be used in driving expression of GP153 in animal models include, without limitation: a tyrosinase promoter or a TRP2 promoter in the case of melanoma cells and melanocytes; an MMTV or WAP promoter in the case of breast cells and/or cancers; a Villin or FABP promoter in the case of intestinal cells and/or cancers; a RIP promoter in the case of pancreatic beta cells; a Keratin promoter in the case of keratinocytes; a Probasin promoter in the case of prostatic epithelium; a Nestin or GFAP promoter in the case of CNS cells and/or cancers; a Tyrosine Hydroxylase, S100 promoter or neurofilament

promoter in the case of neurons; the pancreas-specific promoter described in Edlund et al. Science 230:912-916 (1985); a Clara cell secretory protein promoter in the case of lung cancer; and an Alpha myosin promoter in the case of cardiac cells.

Developmentally regulated promoters may also be selected. They include, without limitation, the murine hox promoters (Kessel et al., Science 249:374-379 (1990)) and the α-fetoprotein promoter (Campes et al., Genes Dev. 3:537-546 (1989)).

Expression Vectors Encoding Peptide Tags

Expression vectors can be designed to fuse the expressed polypeptide to small protein tags that facilitate purification and/or visualization. Many such tags are known and available. Expression vectors can also be designed to fuse proteins encoded by the heterologous nucleic acid insert to polypeptides larger than purification and/or identification tags. Useful protein fusions include those that permit display of the encoded protein on the surface of a phage or cell, fusions to intrinsically fluorescent proteins, such as luciferase or those that have a green fluorescent protein (GFP)-like chromophore, and fusions for use in two hybrid selection systems.

For secretion of expressed proteins, a wide variety of vectors are available which include appropriate sequences that encode secretion signals, such as leader peptides. Vectors designed for phage display, yeast display, and mammalian display, for example, target recombinant proteins using an N-terminal cell surface targeting signal and a C-terminal transmembrane anchoring domain.

A wide variety of vectors now exist that fuse proteins encoded by heterologous nucleic acids to the chromophore of the substrate-independent, intrinsically fluorescent green fluorescent protein from Aequorea victoria ("GFP") and its many color-shifted and/or stabilized variants.

For long-term, high-yield recombinant production of the proteins, protein fusions, and protein fragments of the present invention, stable expression is preferred. Stable expression is readily achieved by integration into the host cell

genome of vectors (preferably having selectable markers), followed by selection for integrants.

GP153 Polypeptides

The present invention relates to full-length GP153 proteins and GP153 protein fragments suitable for use, for example, as antigens, as biomarkers for diseases, and in therapeutic compositions. The invention also relates to fusions of GP153 polypeptides to heterologous polypeptides or conjugation to other moieties.

The invention relates to a full-length GP153 protein (SEQ ID NO:1) optionally having one or more conservative amino acid substitutions. The invention also relates to polypeptide fragments of the GP153 protein, particularly fragments having at least 5, 6, 8, or 15 amino acids of SEQ ID NO:1. Larger fragments of at least 20, 25, 30, 35, 50, 75, 100, 150 or more amino acids are also useful, and at times preferred. The GP153 fragments may be continuous portions of the native GP153 protein. However, knowledge of the complete GP153 amino acid sequence allows a person of skill in the art to recombine various domains that are not contiguous in the native GP153 protein, through application of conventional recombinant DNA technology.

Specific examples of GP153 fragments useful as antigens include, but are not limited to, amino acids 1-704 (extracellular domain, including signal sequence); 30-704 (extracellular domain without signal sequence); 30-280; 30-320; 221-500; 236-488; 409-690; and 500-704 of SEQ ID NO:1. In each of the preceding examples, the GP153 antigen optionally can be conjugated to another moiety or incorporated into a fusion protein, e.g., an Fc fusion, His-tag fusion or GST fusion.

Fusion proteins and conjugates

This invention also relates to fusions of GP153 polypeptides to heterologous polypeptides. As used herein, "fusion" means that the GP153 polypeptide is linearly contiguous to the heterologous polypeptide in a peptide-bonded polymer of amino acids or amino acid analogues. As used herein, "heterologous polypeptide" means a polypeptide that does not naturally occur in contiguity with the GP153 fusion partner. The fusion can consist entirely of a

plurality of fragments of the GP153 protein in altered arrangement. In such a case, any of the GP153 fragments can be considered heterologous to the other GP153 fragments in the fusion protein.

The heterologous polypeptide included within the fusion protein preferably is at least 6 amino acids in length, more preferably at least 8 amino acids in length, and most preferably, at least 15, 20, or 25 amino acids in length. The heterologous sequences can target the GP153 polypeptide to a selected cell by binding to a cell surface receptor, prolong the serum life of the GP153 polypeptide (e.g., an IgG Fc region), make the GP153 polypeptide detectable (e.g., a luciferase or a green fluorescent protein), facilitate purification (e.g., His tag, FLAG, etc.), facilitate secretion of recombinantly expressed proteins (e.g., into the periplasmic space or extracellular milieu for prokaryotic hosts, into the culture medium for eukaryotic cells, through incorporation of secretion signals and/or leader sequences).

Other Modifications of the Polypeptides

Polypeptides used in practicing the invention can be composed of natural amino acids linked by native peptide bonds, or can contain any or all of nonnatural amino acid analogues, nonnative bonds, and post-synthetic (post-translational) modifications, either throughout the length of the polypeptide or localized to one or more portions thereof. However, the range of such nonnatural analogues, nonnative inter-residue bonds, or post-synthesis modifications will be limited to those that do not interfere with the biological function of the polypeptide.

Techniques for incorporating non-natural amino acids during solid phase chemical synthesis or by recombinant methods are well established in the art. For instance, D-enantiomers of natural amino acids can readily be incorporated during chemical peptide synthesis: peptides assembled from D-amino acids are more resistant to proteolytic attack; incorporation of D-enantiomers can also be used to confer specific three dimensional conformations on the peptide. Other amino acid analogues commonly added during chemical synthesis include ornithine, norleucine, phosphorylated amino acids (typically phosphoserine, phosphothreonine, phosphotyrosine), L-malonyltyrosine, a non-hydrolyzable

analog of phosphotyrosine (Kole et al., Biochem. Biophys. Res. Com. 209:817-821 (1995)), and various halogenated phenylalanine derivatives.

Polypeptides used in practicing the invention can include non-native inter-residue bonds. The polypeptides also can include post-translational and post-synthetic modifications, either throughout the length of the protein or localized to one or more portions thereof. For example, when produced by recombinant expression in eukaryotic cells, the polypeptide can include N-linked and/or O-linked glycosylation, the pattern of which will reflect both the availability of glycosylation sites on the protein sequence and the identity of the host cell. Further modification of glycosylation pattern can be performed enzymatically. As another example, recombinant polypeptides may also include an initial modified methionine residue, in some cases resulting from host-mediated processes.

Amino acid analogues having detectable labels are also usefully incorporated during synthesis to provide a labeled polypeptide (e.g., biotin, various chromophores, or fluorophores). The GP153 polypeptides of this invention can also usefully be conjugated to polyethylene glycol (PEG). PEGylation increases the serum half life of proteins administered intravenously for replacement therapy.

Purification of the Polypeptides

Production of the polypeptides optionally can be followed by one or more purification steps. Producing cells include, without limitation, recombinant cells overexpressing the polypeptides, naturally occurring cells (e.g., cancer cells) overxpression the polypeptides, or established cancer cell lines overexpressing the polypeptides. If purification tags have been fused through use of an expression vector that encodes such tags, purification can be achieved, at least in part, by means appropriate to the tags, such as use of immobilized metal affinity chromatography for polyhistidine tags. Other conventional techniques include ammonium sulfate fractionation, immuno-precipitation, fast protein liquid chromatography (FPLC), high performance liquid chromatography (HPLC), and preparative gel electrophoresis. Purification of chemically-synthesized peptides can be achieved, e.g., by HPLC.

A purified protein is present at a concentration of at least 95%, as measured on a mass basis (w/w) with respect to total protein in a composition. Such purities can often be obtained during chemical synthesis without further purification, as, e.g., by HPLC. Purified proteins can be present at a concentration (measured on a mass basis with respect to total protein in a composition) of 96%, 97%, 98%, and even 99%. The proteins even can be present at levels of 99.5%, 99.6%, and even 99.7%, 99.8%, or even 99.9% following purification.

Although high levels of purity are preferred when the isolated proteins are used as therapeutic agents, the isolated proteins are also useful at lower purity. For example, partially purified proteins of the present invention can be used as immunogens to raise antibodies in laboratory animals. The isolated proteins generally are used in substantially purified form. As used herein, "substantially purified protein" means an isolated protein, as above described, present at a concentration of at least 70%, measured on a mass basis with respect to total protein in a composition. Usually, the substantially purified protein is present at a concentration, measured on a mass basis with respect to total protein in a composition, of at least 75%, 80%, or even at least 85%, 90%, 91%, 92%, 93%, 94%, 94.5% or even at least 94.9%.

In preferred embodiments of the invention, the purified and substantially purified proteins are in compositions that lack detectable ampholytes, acrylamide monomers, bis-acrylamide monomers, and polyacrylamide.

Exemplary Uses of GP153 Polypeptides

Certain fragments of at least 8, 9, 10 or 12 contiguous amino acids are also useful as competitive inhibitors of binding of the GP153 protein to its ligand. Such fragments can be used as anti-cancer agents to reduce the activity of GP153.

Fragments of at least six contiguous amino acids are useful in mapping B cell and T cell epitopes of the reference protein. See, e.g., Geysen et al., "Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid," Proc. Natl. Acad. Sci. USA 81:3998-4002 (1984) and U.S. Pat. Nos. 4,708,871 and 5,595,915. Because the fragment need not itself be immunogenic, part of an immunodominant epitope, nor even recognized by native antibody, to be

useful in such epitope mapping, all fragments of at least six amino acids of the proteins of the present invention have utility in such a study.

Fragments of at least eight contiguous amino acids, often at least fifteen contiguous amino acids, have utility as immunogens for raising antibodies that recognize the proteins of the present invention or as vaccines for GP153 -mediated diseases such as cancers.

The GP153 proteins, fragments, and fusions of the present invention can be usefully attached to a substrate. When bound to a substrate, the polypeptides can be used to detect and quantify antibodies, e.g., in serum, that bind specifically to the immobilized protein.

Antibodies and Antibody-Producing Cells

General Considerations

The invention relates to antibodies that bind specifically to the GP153 polypeptides. The antibodies can be specific for linear epitopes, discontinuous epitopes, or conformational epitopes of such polypeptides, either as present on the polypeptide in its native conformation or, in some cases, as present on the polypeptides as denatured, as, e.g., by solubilization in SDS. In some embodiments, the invention provides antibodies, both polyclonal or monoclonal, that bind specifically to a polypeptide having an amino acid sequence presented in SEQ ID NO:1.

As used herein, "antibody" means a full antibody, e.g., an antibody comprising two heavy chains and two light chains, or to an antigen-binding fragment of a full antibody. Such fragments include, but are not limited to, those produced by digestion with various proteases, those produced by chemical cleavage and/or chemical dissociation, and those produced recombinantly, so long as the fragment remains capable of specific binding to an antigen. Among these fragments are Fab, Fab', $F(ab')_2$ and single chain Fv (scFv) fragments.

An antibody can be a murine or hamster antibody or a homolog thereof, or a fully human antibody. An antibody can also be a humanized antibody, a chimeric antibody, an antibody fusion, a diabody, an intrabody, or a single-chained

antibody. An antibody can be of any isotype and subtype, for example, IgA (e.g., IgA1 and IgA2), IgG (e.g., IgG1, IgG2, IgG3 and IgG4), IgE, IgD, IgM, wherein the light chains of the immunoglobulin may be of type kappa or lambda. While monoclonal antibodies are generally preferred, polyclonal antibodies, e.g., from mice, rabbits, turkeys, or sheep, also may be used.

Typically, the affinity or avidity of an antibody (or antibody multimer, as in the case of an IgM pentamer) will be at least 1 x 10^{-6} molar (M), preferably at least 5 x 10^{-7} M, more preferably at least 1 x 10^{-7} M, with affinities and avidities of at least 1 x 10^{-8} M, 5 x 10^{-9} M, and 1 x 10^{-10} M being especially useful.

Moieties Conjugated to the Antibodies

Antibodies are useful in various *in vitro* immunoassays, such as western blotting and ELISA, in isolating and purifying GP153 proteins (e.g., by immunoprecipitation, immunoaffinity chromatography, and magnetic bead-mediated purification). The antibodies are also useful as modulators (i.e., antagonists or agonists) of a GP153 protein *in vivo* to modulate the protein's interaction with its natural ligand. The antibodies can also be used to conjugate to cytotoxic reagents for *in vivo* targeted delivery.

Anti-GP153 antibodies can be associated with moieties appropriate for their uses. When the antibodies are used for immunohistochemical staining of tissue samples, the moieties can be an enzyme that catalyzes production and local deposition of a detectable product. Exemplary enzymes are alkaline phosphatase, β-galactosidase, glucose oxidase, horseradish peroxidase (HRP), and urease. The antibodies also can be labeled using colloidal gold. When the antibodies are used, e.g., for flow cytometric detection and scanning laser cytometric detection, they can be labeled with fluorophores. For secondary detection using labeled avidin, streptavidin, captavidin or neutravidin, the antibodies of the present invention can be labeled with biotin. The antibodies also can be labeled with radioisotopes. When the antibodies are to be used for *in vivo* diagnoses, they can be rendered detectable by conjugation to MRI contrast agents, such as radioisotopic labeling or gadolinium diethylenetriaminepentaacetic acid (DTPA).

The antibodies also can be conjugated to cytotoxic agents for targeting the agents to a tumor site. For example, the antibody can be conjugated to *Pseudomonas* exotoxin A, diphtheria toxin, shiga toxin A, anthrax toxin lethal factor, or ricin. See, e.g., Hall (ed.), Immunotoxin Methods and Protocols (Methods in Molecular Biology, Vol 166), Humana Press (2000) (ISBN:0896037754); and Frankel et al. (eds.), Clinical Applications of Immunotoxins, Springer-Verlag New York, Incorporated (1998) (ISBN:3540640975). Small molecule toxins such as calicheamycin or chemotherapeutic agents also can be delivered via chemical conjugation to the antibodies. The antibodies also can be used to deliver DNA to a tumor site as gene therapy to inhibit or otherwise modify the behavior of the tumor, e.g., to deliver an antisense reagent to the GP153 oncogene.

For some uses, the antibodies can be bound to a substrate via a linker moiety. For example, the antibodies can usefully be conjugated to filtration media, such as NHS-activated Sepharose or CNBr-activated Sepharose for immunoaffinity chromatography. The antibodies can also be attached to paramagnetic microspheres, e.g., by biotin-streptavidin interaction. The microsphere then can be used for isolation of cells that express or display the protein of interest. As another example, the antibodies can also be attached to the surface of a microtiter plate for ELISA.

Nucleic Acid Aptamers

Aptamers for GP153 are DNA-based or RNA-based molecules that bind to GP153 proteins with high affinity and specificity. Large libraries of nucleic acid compounds can be screened to identify specific molecules that e.g., bind GP153 polypeptides, or inhibit GP153 enzymatic activity. Like antibodies and small molecules, they can be used as research tools or therapeutic agents. For a review, see Rimmele, Chembiochem 4:963-71 (2003)).

Pharmaceutical Compositions

The invention relates to pharmaceutical compositions comprising antagonists of GP153 activity, and methods of using them to prevent or treat (i.e.,

ameliorate, mitigate, alleviate, slow, or inhibit) tumor growth, angiogenesis, metastasis or otherwise inappropriate cell proliferation.

Inhibitors of GP153 activity also can be used in combination with one or more other therapeutic agents, for improved cancer treatment. Other therapeutic agents suitable for co-administration with a GP153 inhibitor include, for example, an anti-angiogenic agent, an anti-metastatic agent, or an agent used to create a hypoxic environment. Chemotherapeutic agents that can be co-administered with inhibitors of GP153 include folate antagonists, pyrimidine and purine antimetabolites, alkylating agents, platinum antitumor compounds, DNA interchelators, other agents that induce DNA damage, microtubule targeting products, small molecule inhibitors of protein kinases and biological inhibitors of growth factor receptors. The GP153 inhibitor and additional therapeutic agent(s) may be used concurrently or sequentially. In some embodiments, the subject is pre-treated with one or more agents, followed by treatment with a GP153 inhibitor.

The ability of tumor cells to detach from the primary site and produce metastases in a distant organ is due to the survival and growth of a unique subpopulation of cells with metastatic properties. Tumor growth and metastasis are angiogenesis-dependent. Inhibition of angiogenesis will generate a hypoxic environment in the tumor, forcing tumor cells to become dependent upon the glycolytic pathway for energy production. Therefore, preventing angiogenesis in combination with inhibiting GP153 is a promising therapeutic strategy. Angiogenesis inhibitors (e.g. angiostatin, endostatin, Avastin™ or VEGF trap technology) can be used in combination with GP153 inhibitors as an effective anti-cancer therapy. Such a combination can be expected to have a synergistic effect. This may also allow the use of a lower dose of GP153 inhibitor or anti-angiogenic agent or both in chemotherapy. This is desirable because it is likely to cause less toxicity in patients. In addition, the use of combinations of therapeutic agents may circumvent drug resistance problems.

GP153 inhibitors also can be used in combination with agents that create a hypoxic environment to enhance the effect of GP153 inhibitor. Hypoxia, i.e., lack of oxygen, plays a fundamental role in many pathologic processes. In response to hypoxia, mammalian cells activate and express multiple genes. Tumor cells may

respond to hypoxia by diminishing their proliferative rates leaving the cells viable but nonproliferating. Some transformed cell lines can also undergo apoptosis in extreme hypoxia and an acidic environment. Similar to inhibitors of angiogenesis, agents that induce a hypoxic environment may sensitize tumor cells to inhibition of GP153 and use of hypoxia inducing agents in combination with inhibiting GP153 is therefore another promising therapeutic strategy.

An increase in apoptosis (programmed cell death) has been associated with a decrease in tumor proliferation. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in several human tumors. A number of cell regulatory pathways such as Rb/E2F pathway, the c-Myc transcription factor, and the Ras signaling molecule have also been shown to control not only cell proliferation but also pathways leading to apoptosis. Further, a combination of GP153 antagonist or inhibitor with reagents that activate apoptotic signals, or inhibit survival signals, can also be used for cancer therapy. Survival signals that recently have been shown to modulate apoptotic signaling include the focal adhesion kinase (FAK), the phosphinositol 3' kinase (PI3'K), and protein kinase B (PKB, also known as Akt).

A composition of the invention typically contains from about 0.1 to 90% by weight (such as 1 to 20% or 1 to 10%) of a therapeutic agent of the invention in a pharmaceutically accepted carrier. Solid formulations of the compositions for oral administration can contain suitable carriers or excipients, such as corn starch, gelatin, lactose, acacia, sucrose, microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, calcium carbonate, sodium chloride, or alginic acid. Disintegrators that can be used include, without limitation, microcrystalline cellulose, corn starch, sodium starch glycolate, and alginic acid. Tablet binders that can be used include acacia, methylcellulose, sodium carboxymethylcellulose, polyvinylpyrrolidone(Povidone™), hydroxypropyl methylcellulose, sucrose, starch, and ethylcellulose. Lubricants that can be used include magnesium stearates, stearic acid, silicone fluid, talc, waxes, oils, and colloidal silica.

Liquid formulations of the compositions for oral administration prepared in water or other aqueous vehicles can contain various suspending agents such as methylcellulose, alginates, tragacanth, pectin, kelgin, carrageenan, acacia,

polyvinylpyrrolidone, and polyvinyl alcohol. The liquid formulations can also include solutions, emulsions, syrups and elixirs containing, together with the active compound(s), wetting agents, sweeteners, and coloring and flavoring agents. Various liquid and powder formulations can be prepared by conventional methods for inhalation into the lungs of the mammal to be treated.

Injectable formulations of the compositions can contain various carriers such as vegetable oils, dimethylacetamide, dimethylformamide, ethyl lactate, ethyl carbonate, isopropyl myristate, ethanol, polyols (glycerol, propylene glycol, liquid polyethylene glycol, and the like). Physiologically acceptable excipients can include, for example, 5% dextrose, 0.9% saline, Ringer's solution or other suitable excipients. Intramuscular preparations, e.g., a sterile formulation of a suitable soluble salt form of the compounds, can be dissolved and administered in a pharmaceutical excipient such as Water-for-Injection, 0.9% saline, or 5% glucose solution. A suitable insoluble form of the compound can be prepared and administered as a suspension in an aqueous base or a pharmaceutically acceptable oil base, such as an ester of a long chain fatty acid (e.g., ethyl oleate).

A topical semi-solid ointment formulation typically contains a concentration of the active ingredient from about 1 to 20%, e.g., 5 to 10%, in a carrier such as a pharmaceutical cream base. Various formulations for topical use include drops, tinctures, lotions, creams, solutions, and ointments containing the active ingredient and various supports and vehicles. The optimal percentage of the therapeutic agent in each pharmaceutical formulation varies according to the formulation itself and the therapeutic effect desired in the specific pathologies and correlated therapeutic regimens.

Pharmaceutical formulation is a well-established art, and is further described in Gennaro (ed.), Remington: The Science and Practice of Pharmacy, 20th ed., Lippincott, Williams & Wilkins (2000) (ISBN: 0683306472); Ansel et al., Pharmaceutical Dosage Forms and Drug Delivery Systems, 7th ed., Lippincott Williams & Wilkins Publishers (1999) (ISBN: 0683305727); and Kibbe (ed.), Handbook of Pharmaceutical Excipients American Pharmaceutical Association, 3rd ed. (2000) (ISBN: 091733096X). Conventional methods, known to those of

skill in the art can be used to administer the pharmaceutical formulation(s) to the patient.

A transdermal pharmaceutical formulation can be administered to a patient by applying to the skin of the patient a transdermal patch containing the pharmaceutical formulation, and leaving the patch in contact with the patient's skin (generally for 1 to 5 hours per patch). The pharmaceutical formulation(s) can also be administered via other conventional routes (e.g., parenteral, subcutaneous, intrapulmonary, transmucosal, intraperitoneal, intrauterine, sublingual, intrathecal, or intramuscular routes) by using standard methods. In addition, the pharmaceutical formulations can be administered to the patient via injectable depot routes of administration such as by using 1-, 3-, or 6-month depot injectable or biodegradable materials and methods.

Regardless of the route of administration, the therapeutic protein or antibody agent typically is administered at a daily dosage of 0.01 mg to 30 mg/kg of body weight of the patient (e.g., 1mg/kg to 5 mg/kg). The pharmaceutical formulation can be administered in multiple doses per day, if desired, to achieve the total desired daily dose. The effectiveness of the method of treatment can be assessed by monitoring the patient for known signs or symptoms of a disorder.

The pharmaceutical compositions of the invention may be included in a container, package or dispenser alone or as part of a kit with labels and instructions for administration. These compositions can also be used in combination with other cancer therapies involving, e.g., radiation, photosensitizing compounds, anti-neoplastic agents and immunotoxics.

Non-Human Mammals Genetically Modified with Respect to GP153

General Considerations

This invention provides genetically modified nonhuman transgenic mammals, e.g., mice, at least some of whose cells whose somatic cells express a recombinant gene encoding GP153. Such mammals can be used to study the effect of GP153 expression on tumorigenicity and tumor development, to study the role of GP153 on normal tissue development and differentiation, to identify via array CGH regions of the genome whose amplification or deletion is correlated with

GP153 status, and to screen for and establish toxicity profiles of anti-cancer drugs. This invention also provides transgenic non-human mammals with targeted disruption of one or both copies of the endogenous GP153 gene. Animal models according to the invention can be conventional germline transgenic animals or chimeric animals.

Inducible Cancer Model

This invention provides an inducible cancer model to study tumor biology and to screen for anti-cancer drugs. In some embodiments, the inducible cancer model is a mouse whose genome has been modified to include: (a) an expression construct comprising a GP153 coding sequence operably linked to an inducible promoter, and (b) a genetic mutation that causes the mouse to have greater susceptibility to cancer than a mouse not comprising the genetic mutation. In the model, expression of the GP153 coding sequence leads to development of cancer in the transgenic mouse. The cancer regresses when expression of the GP153 coding sequence is reduced or eliminated. Mutations that render the animal more susceptible to cancer include disabling mutations in a tumor suppressor gene (e.g., INK4a), disabling mutations in a DNA repair gene (e.g., MSH2), and activating mutations in an oncogene (e.g., *myc* and *ras*). Such testing also can be carried out in cells (e.g., human cells) that are engineered to contain an inducible oncogene and endowed with tumorigenic capacity by the presence of an appropriate combination of oncogenes, tumor suppressor genes, and/or telomerase.

In one particular embodiment, the animal's genome comprises (i) a first expression construct containing a gene encoding a reverse tetracycline transactivator operably linked to a promoter, such as any tissue or cell type-specific promoter or any general promoter, and (ii) a second expression construct containing the GP153 coding sequence operably linked to a promoter that is regulated by the reverse tetracycline transactivator and tetracycline (or a tetracycline analogue, for example, doxycycline). The mammal is observed with and without administration of the tetracycline (or tetracycline analogue) for the development, maintenance, or progression of a tumor that is tetracycline-dependent. Other inducible systems such as those described can also be employed.

This animal model can be used to determine the efficacy of a candidate compound in preventing or treating cancer. This method involves administering to the animal a candidate compound and observing the effect of the compound on tumor development, maintenance, angiogenesis and/or progression in the animal. Regression and/or reduction of tumor size in the presence of the compound is indicative of the effectiveness of the compound. Similarly, the effect of a candidate compound on the level of GP153 mRNA, protein, or activity in the animal or cell lines derived from the mammal (or cell lines transfected with the gene) can be used to identify the candidate as an agonist or antagonist. The ability to compare the effect of a test compound to that of genetically switching off the inducible oncogene in this system allows the identification of surrogate markers that are predictive of the clinical response to the compound. The inducible model can be used to determine whether a compound can eradicate minimal residual tumor. Normally in the inducible model, a tumor regresses when the GP153 gene is switched from "on" to "off" via the inducible promoter. But if a compound can eradicate minimal residual tumor, switching the gene back on after administration of the compound will not bring back the tumor.

The animal model can also be used to identify other cancer-related elements. To do this, a detailed expression profile of gene expression in tumors undergoing regression or regrowth due to the inactivation or activation of the GP153 transgene is established. Techniques used to establish the profile include the use of suppression subtraction (in cell culture), differential display, proteomic analysis, serial analysis of gene expression (SAGE), and expression/transcription profiling using cDNA and/or oligonucleotide microarrays. Then, comparisons of expression profiles at different stages of cancer development can be performed to identify genes whose expression patterns are altered.

This animal model can also be used to identify molecular surrogates of GP153 in another manner. To do this, the expression of the GP153 gene is turned off by removal of the inducer, and another round of MaSS screening is performed using retroviral integration, cDNA complementation, or the genetic supressor elements (GSE) method. Genes whose activation results in transformation of the cells are likely to be in a related tumorigenic pathway as GP153.

The animal model also can be used to identify surrogate biomarkers for diagnosis or for following disease progression in patients. The biomarkers can be identified based on the differences between the expression profiles of the "on" and "off" states in the animal model. Blood or urine samples from the animal can be tested with ELISAs or other assays to determine which biomarkers are released from the tumor into circulation during tumor genesis, maintenance, or regression (when GP153 is turned off). These biomarkers are particularly useful clinically in following disease progression post anti-GP153 therapy.

Cancer Diagnostics

Since GP153 expression and/or activity is up-regulated in tumor cells, one can use GP153 as a marker in diagnosing cancer or any other abnormal hyperplasia conditions. To do this, the above-described nucleic acid probes or antibodies are used to quantify the expression level of GP153 in a tissue sample. An increase in that level relative to control is indicative of cancerous, neoplastic, or hyperplastic pathology of the tissue sample. This type of test can be performed using conventional techniques such as RT-PCR, ribonuclease protection assays, *in situ* hybridization, Northern blot analysis, FISH, CGH, array CGH, SKY, or immunohistochemistry.

Because up-regulation of GP153 is generally associated with a malignant state, a GP153 polypeptide may be elevated in a tissue sample (e.g., blood or urine) of cancer patients relative to that of normal individuals. This elevation can be detected, e.g., by ELISAs, radioimmunoassays, or protein chip assays. Such tests may not be useful only for diagnosis of GP153 -related diseases such as cancers, but also for monitoring the progress of therapy using GP153 inhibitors.

EXAMPLES

The invention is further illustrated by the following examples. The examples are provided for illustrative purposes only, and are not to be construed as limiting the scope or content of the invention in any way.

Example 1: MaSS Screening Identification of the Gene

This example describes the procedures for identifying the GP153 gene by MaSS screening.

Mo-MuLV producer cell line TMJ (NIH3T3 based cell line) was plated to the required number of plates (100 mm). These cells were cultured and maintained in RPMI media with 10% FBS. For viral production, TMJ cells were fed with 4-5 ml of fresh culture media, and culture supernatant was harvested 8-12 hours later. The supernatant was filtered through a 0.45 μM filter.

Meanwhile, doxycycline-dependent, RAS-induced melanoma cells (R545 cells) were maintained in RPMI media with 10% fetal calf serum in the presence of doxycycline (2 μg/ml). At approximately 18-24 hrs after plating or when the plates were 70-80% confluent, the R545 cells were infected with the filtered viral supernatant in the presence of polybrene (6-8 μg/ml). From this point on, the R545 cells were maintained in the absence of doxycycline.

Eighteen hours after infection, infected R545 cells were trypsinized, rinsed and resuspended in Hanks' Balanced Salt Solution. Cell suspensions were kept on ice and the handling time after trypsinization was kept to a minimum. About 1 X 10^6 cells were injected onto the flank of SCID mice fed with water without doxycycline. The animals were observed for tumor development. Control animals were similarly injected with 1 X 10^6 uninfected R545 cells. Tumors typically developed after approximately 21 days. Tumors were harvested and tumor tissues were immediately snap-frozen in liquid nitrogen.

DNA was isolated from tumor tissues using the PUREGENE™ DNA isolation kit. 10 µg of genomic DNA was digested to completion with either BamHI or SacII and the reaction was terminated by incubation at 65°C for 20 minutes. The digested samples were self-ligated in a diluted 600 µl reaction volume using 4000 U of high concentration T4 Ligase (NEB, Cat. # M0202M). The ligation was performed overnight to 24 hrs at 16°C. The ligated DNA was precipitated with ethanol and dissolved in 40 µl of sterile water. The ligated DNA was then serially diluted to 1:10 and 1:100 ratios and subjected to inverted polymerase chain reaction (IPCR).

The PCR reaction mix had a total volume of 50 μl and contained 1μl of the ligated DNA, 25 nmol of each dNTP, 10 pmol each of the forward and reverse primers, 1 X Buffer 2, and 2.5 U of Enzyme Mix in the EXPAND Long Template PCR System (Roche). Amplification was performed as follows: 92°C for 2 min, then 10 cycles of (92°C for 10 sec, 63° for 30 sec, 68°C for 15 min), then 20 cycles of (92°C for 10 sec, 63°C for 30 sec, 68°C for 15 min, and a 20 sec auto extension), and a final extension step at 68°C for 30 min (TETRAD™ Thermocycler, MJ Research). The primer sets used in IPCR (all of them targeting the retroviral LTRs) were:

S5'1F: GAGGCCACCTCCACTTCTGAGAT (SEQ ID NO:15);

S5'1R: CTCTGTCGCCATCTCCGTCAGA (SEQ ID NO:16);

S5'2F: CAUCAUCAUCAUCCTGCCCCCTCTCCCATAGTGT (SEQ ID NO:17);

S5'2R: CUACUACUACUAGGCGTTACTGCAGTTAGCTGGCT (SEQ ID NO:18);

S3'1F: GGCTGCCATGCACGATGACCTT (SEQ ID NO:19);

S3'1R: CGGCCAGTACTGCAACTGACCAT (SEQ ID NO:20);

S3'2F: CUACUACUACUAGGGAGGGTCTCCTCAGAGTGATT (SEQ ID NO:21);

S3'2R: CAUCAUCAUCAUGGAAAGCCCGAGAGGTGGT (SEQ ID NO:22);

B3'1R: CGGGAAGGTGGTCGTCGGTCT (SEQ ID NO:23); and

B3'2R: CAUCAUCAUCAUGGGGCCCCGAGTCTGTAATTT (SEQ ID NO:24).

For BamHI 5' cloning, primary PCR was done by using S5'1F and S5'1R followed by nested PCR using S5'2F and S5'2R. For BamHI 3' cloning, primary PCR was done by using S3'1F and B3'1R followed by nested PCR using S3'2F and B3'2R. For SacII 5' cloning, primary PCR was done by using S5'1F and S5'1R followed by nested PCR using S5'2F and S5'2R. For SacII 3' cloning, primary PCR was done by using S3'1F and S3'1R followed by nested PCR using S3'2F and S3'2R.

The PCR products were resolved on 1% agarose gel. Individual bands were excised and purified using the QIAGEN Gel Extraction kit. The purified DNA was dissolved in 30 μl of sterile water and subjected to direct DNA sequencing.

The site of retroviral integration into the mouse genome was mapped for all IPCR sequences as follows. Retroviral leader sequences were trimmed from the raw sequences of IPCR products, and homology searches for the trimmed sequences were performed in the NCBI MGSCV3 database by using the BLAST software program. BLAST hits were analyzed and recurrent sites of integration in multiple mouse tumors were identified. Recurrence was defined as 2 or more integrations within a 10 kb region. To identify genes whose expression was affected by the retroviral integration, NCBI MapView was used to identify the site of each recurrent retroviral integration onto the mouse genome. Genes immediately neighboring the site were identified by using the MGSCV3 Gene map. These genes were defined as candidate cancer-related genes because in the vast majority of cases, MuLV integration affects the most proximal genes. When the integration occurred within a gene, that gene was deemed the best candidate as the target for the effects of retroviral integration.

Example 2: Expression in Human Tumors and Tumor Cell Lines

This example describes the protocols for expressing the candidate gene identified above in human cancer cells.

Primer pairs for each human gene are designed as described for the mouse gene. Expression of each candidate gene is assessed in a panel of 31 human cancer cell lines and 47 human primary tumors by using real-time reverse transcription PCR. The forward and reverse primers are 5'- ccatgattagcaggccttatagc -3' (SEQ ID NO:25) and 5'- ccaggtcaaacaactctgcaaa -3' (SEQ ID NO:26), respectively. RNA is prepared from the cells using QIAGEN RNEASY™ mini-prep kits and QIAGEN RNEASY maxi-prep columns. RNA preparations are treated with DNase during column purification according to manufacturer's instructions. Expression of each gene is determined in triplicate for all tumors and cell lines using SYBR green-based real-time PCR. To do this, 2X SYBR green PCR master

mix (ABI) is mixed with the MULTISCRIBE™ reverse transcriptase (ABI) and RNase inhibitor (ABI). Forward and reverse primers are added at ratios previously optimized for each gene using control human reference RNA (Stratagene). 50 ng of RNA template is used per reaction, and the reactions are performed in a total volume of 20 µl. Real-time quantification is performed using the ABI 7900HT and SDS2.0 software. RNA loading is normalized for β-actin and 18S rRNA. RNA quantity is determined relative to human universal reference RNA (Stratagene) to permit run-to-run comparisons.

Example 3: Inhibition of Human Cancer Cell Lines by siRNA

This example describes the protocols used to inhibit the expression of the candidate gene in human cancer cells by siRNA.

Double-stranded siRNA oligonucleotides were designed using the OligoEngine siRNA design tool (http://www.oligoengine.com). HPLC purified siRNA oligonucleotides were incubated at 95°C for 1 minute, 37°C for 1 hour, and room temperature for 30 minutes. The siRNAs were stored at -20°C prior to use.

Human cancer cell lines were transfected with siRNA using the Oligofectamine reagent from Invitrogen. Transfected cells were allowed to grow for at least 24 hours. The cells were then trypsinized and reseeded for growth curve analysis or growth in soft agar.

To create growth curves, early passage cell lines were seeded at 2 X 10^4 cells per well in 12-well plates. At 6 (Day 0), 24 (Day 1), 48 (Day 2), 72 (Day 3), 96 (Day 4) and 120 hours (Day 5) after plating, duplicate plates of cells were washed, fixed in 10% buffered formalin, and stained with crystal violet for 30 minutes at room temperature. Stained cells were washed in double distilled water, and stain was extracted using 10% acetic acid. Absorbance of the extracted stain was read at 590 nm. The mean absorbance per well of duplicate cultures was determined.

Colony formation assay. One day prior to seeding cells for assay, 6-well plates containing bottom agar were prepared. The bottom agar was made of 0.7% agarose (SeaKem GTG agarose) and 1X DME plus 10% fetal bovine serum. The next day, cells were seeded into each well by adding 5 ml of a mixture containing 1

X 10^4 cells in 0.32% agarose and 1 X DME plus 10% FBS. The cell mixture was allowed to solidify at room temperature for 30 minutes. The plates were then incubated at 37°C in a 5% CO_2 atmosphere for 1 to 14 days. Colony formation was analyzed and photographs taken at various time points.

Apoptosis assay. Twenty-four hours after transfection with siRNA, cells were plated in quadruplicate in 96-well plates at appropriate densities. Forty-eight hours after plating of cells (72 hours post-transfection) cells were assayed for apoptosis using an ELISA assay to detect free nucleosomes resulting from DNA cleavage, a result of apoptosis. The ELISA assay was performed using the Cell Death Detection ELISA Plus™ kit (Roche catalog no. 1920685) according to the vendor's instructions.

The effect of transfecting GP153-specific siRNAs in human cancer cell lines DLD-1 (colon), A549 (lung), SW620 (colon), LNCAP (prostate), HCT-116 (colon), HCT-115 (colon), BT474 (breast), ACHN (renal), U251 (glioblastoma) and SKOV-3 (ovarian carcinoma) was compared to the effect of transfecting a negative control siRNA that targets firefly luciferase. The data are summarized in Table 1.

Table 1: siRNA Soft Agar Assay Results

	siRNA	Expression (%)	Apoptosis (fold)	Colonies (%)
DLD-1	1	10	n/c	65
	2	11	n/c	99
	3	15	n/c	12
A549	1	5	n/c	100
	2	8	n/c	98
	3	10	n/c	35
SW620	1	12	1.5x	25
	2	21	n/c	40
	3	21	1.4x	40
LNCAP	1	10	1.8	20
	2	12	1.9	20
	3	15	n/c	20
HCT-116	1	20	2.0x	50
HCT-15	1	40	n/c	90
BT474	1	20	n/c	N/A
ACHN	1	20	n/c	100
U251	1	2	1.7x	55

SKOV3	1	38	n/c	90

Values shown for expression indicate the level of expression of GP-153 in cells transfected with the siRNA to GP-153 as a percentage of the level of expression of GP-153 in cells transfected with the negative control siRNA. Values shown for apoptosis indicate the level of apoptosis of GP-153 in cells transfected with siRNA to GP-153 relative to the level of apoptosis in cells transfected with the negative control siRNA. Values shown for colony growth in soft agar indicate the number of colonies formed in soft agar by cells transfected with siRNA to GP-153 as a percentage of the number of colonies formed in soft agar by cells transfected with the negative control siRNA. The symbol "n/c" indicates no change as compared to the negative control.

Significant inhibition of growth in soft agar was observed in SW620 colon carcinoma and LNCAP prostate carcinoma cell lines upon inhibition of GP-153 expression with 3 independent siRNAs. No significant inhibition of growth in soft agar was observed in 8 other human cancer cell lines tested. This suggested that GP-153 is essential for tumorigenesis and/or tumor maintenance in specific tumor settings.

Growth curve experiments also can be performed in a similar manner to the experiment described above, but in a hypoxic chamber under conditions where the oxygen level is reduced to 0.2 – 1.0%. Growth of cells under hypoxic conditions in the face of inhibition of GP153 by siRNA is assessed at 6 (Day 0), 24 (Day 1), 48 (Day 2), 72 (Day 3), 96 (Day 4) and 120 hours (Day 5) after plating.

Example 4: MEF Transformation

This example describes the protocols for using the candidate gene to transform mouse embryonic fibroblasts.

MEFs are isolated from 100 individual 13.5-day-old embryos. The isolated MEFs are pooled and grown in DMEM supplemented with 10% FBS, penicillin and streptomycin. Pooled early-passage (passages 4 to 6) $p16^{Ink4a}/p19^{Arf}$ -/- mouse embryonic fibroblasts are transfected with: (1) pKO-Myc; (2) pKO-Myc and pT24-RasV12; (3) pKO-Myc and GP153 cDNA (GeneCopeia); or (4) pKO-Myc, pT24-RasV12, and GP153 cDNA (GeneCopeia). All transfections are done in duplicate

cultures (8 X 10^5 cells) using Lipofectamine Plus™ in Optimem (Gibco) without serum or antibiotics. Cultures are split 1:3 a day after transfection. Foci are counted on day 12.

Example 5: Inhibition of Tumor Growth by si RNA in Tumor Explant Models

Human cancer cell lines are infected with a retrovirus driving constitutive expression of luciferase. Stable cell lines expressing high levels of luciferase are selected using antibiotic resistance markers in the integrated retrovirus. These cell line are then transfected with siRNA using the Oligofectamine reagent from Invitrogen. Transfected cells are allowed to grow for at least 24 hours. The cells are then trypsinized and resuspended in Hank's Buffered Salt Solution. One million cells are injected subcutaneously into the flanks of BalbC nude mice. Mice are imaged daily to determine the size of each tumor based upon the level of luciferase detected, an indicator of the number of tumor cells present. Imaging is performed by intra-peritoneal injection of 250 μg of luciferin per gram of mouse weight. Luminescence is detected using a cooled CCD camera and quantification of image intensity, a measure of tumor size, is performed using Winlight 32 software from Berthold.

Example 6: Effect of shRNA on Human or Mouse Cancer Cell Lines in SCIDs

Human cancer cell lines expressing high levels of GP153, or cell lines established from the inducible mouse cancer model described above, are transfected with a vector encoding a short hairpin RNA (shRNA) homologous to GP153 gene. Expression of the shRNA is placed under the control of an inducible U6 promoter. Stable cell lines are established. Approximately 5 X 10^5 cells are injected subcutaneously into the flank of 6 week old female inbred SCID mice. Tumor formation is observed visually. For cell lines derived from the inducible mouse model, tumor formation is induced by doxycycline. Expression of the shRNA is induced once tumors were visually identified. In the case of mouse model-derived tumor cells, the mice are fed with doxycycline. Tumor regression is followed using calipers to measure the shrinking tumor diameter.

Example 7: Tumor Formation in SCIDs

Tumor cells derived from the tumor suppressor null (INK4/arf -/-) doxycycline-inducible oncogene mouse model are infected with retrovirus encoding the candidate gene under the control of an IPTG-inducible promoter. Stable cell lines are established. 10^6 cell are injected subcutaneously into the flank of 6 week old female inbred SCID mice. Mice are fed with doxycycline for 7-12 days. 24 hours prior to doxycycline withdrawal, mice are fed IPTG. IPTG feeding is maintained after withdrawal of doxycycline and tumor regression is monitored using calipers.

Example 8: Effect of Anti-GP153 Antibody on Human Cancer Cell Lines

A panel of human cancer cell lines is examined for expression of mRNAs encoding GP153. The human cancer cells are cultured in 10 cm tissue culture dishes until 95% confluence. The total cellular RNA is harvested using Trizol (Invitrogen) and used in real time PCR for determination of mRNA expression. Specific PCR primers are designed using Primer 3 software (Whitehead Institute). The quantitative real time PCR is conducted in an ABI Prism 7900HT Sequence Detection System (AB Applied Biosystems) using human beta-2 microglobulin as internal control. The relative expression of mRNA is derived using the software of the equipment and calculated with Microsoft Excel. Human cancer cell lines expressing GP153 mRNA are chosen for further testing with anti-GP153 antibody.

To test the effect of anti-GP153 antibody on the proliferation and colony formation of human cancer cells, soft agar and MTT assays are used. For both soft agar and MTT assays, human cancer cells are inoculated into 12-well tissue culture plates at densities of 20,000 to 50,000 cells/well and incubated at 37°C for 24 to 40 hours. Then, the cells in the plates are treated with different concentrations of antibody for 48 hours. After antibody treatment, the cells are harvested by trypsinization and inoculated into soft agar or fresh 24-well plates for soft agar and MTT assay, respectively.

For the soft agar assay, antibody-treated cells in soft agar are incubated at 37°C for 5 days. Fresh media containing antibody are added to the wells on day 3 and the colonies are counted on day 5.

For the MTT assay, antibody-treated cells in 24-well plates are incubated at 37°C for 48 hours. After washing the cells with PBS, fresh media containing MTT are added to the wells and the plates are incubated at 37°C for an additional 24 hours. The amount of MTT converted by living cells is determined by photo-spectrometry.

Example 9: GP153 shRNA competition in vivo

The tumor maintenance function of GP153 was validated *in vivo* using an inducible shRNA system and tumor xenografts. The inducible shRNA system employed was an *in vivo* RNAi competition assay. In this assay, HCT116/Tet repressor cells individually expressing approximately a dozen shRNAs against genes of interest were mixed and injected into SCID mice. The mice then were divided into two groups: one group received doxycycline to induce the expression of shRNAs and the other group did not. The representation of the cells expressing each individual shRNA in the tumor samples harvested was measured by quantitative PCR analysis. If the abundance of cells expressing a specific shRNA decreases in tumors exposed to doxycycline compared to the abundance of cells expressing that shRNA in tumors not exposed to doxycycline, the target gene has an essential role in tumor viability or maintenance. If the abundance of a particular shRNA in tumors is increased upon doxycycline treatment, the target gene has a negative role in tumor growth. If the abundance is unchanged, the gene has no effect on tumor growth.

In the experiment summarized in Table 2, HCT116/Tet repressor cells were first engineered to express an shRNA against a gene of interest as previously described. These cells were cultured in vitro separately in DMEM with 10% Fetal Bovine Serum, and then harvested and mixed in equal ratio. The mixed population of cells was injected into 20 SCID mice (10e+6 cells per injection, 2 injection sites per mouse). The mice were then divided into two groups: one group received doxycycline at day 6 to induce the expression of shRNAs and the other group did not. The tumors were collected at day 26. The average weight of the tumors without doxycycline treatment was 0.236 g (n=8) and that with doxycycline treatment was 0.212 g (n=11). Upon pulverization of the tumors, the DNA was extracted (Qiagen genomic DNA extraction kit) followed by quantitative PCR with

SYBR green (Qiagen) to examine the abundance of the shRNA hairpins using a vector specific primer (pLentiTO2B_1, CTCGACGGTATCGCTAGTCC (SEQ ID NO:27) and a hairpin-specific primer (Table 2). During the quantitative PCR cycle, the polymerase was first activated at 95°C for 15 minutes followed by 40 cycles of denaturation at 95°C, annealing at 54°C and elongation at 72°C. The representation of the cells expressing each individual shRNA (subpopulation) in the tumor samples was then calculated. In this experiment, the average abundance of the cells expressing PTK-7 hairpin 1 or 2 was decreased by 95.8% or 88% respectively in tumors exposed to doxycycline compared to that in untreated tumors, indicating that PTK7 has an essential role in tumor viability or maintenance. For comparison, it can be seen in Table 2 that the average abundance of the cells expressing the K-Ras hairpin decreased by 96.8% or in tumors exposed to doxycycline, but the average abundance of cells expressing the K-Ras hairpin decreased by negative 22.5%, i.e., increased by 22.5% in tumors not exposed to doxycycline, i.e., tumors in which expression of the hairpin was not induced.

Table 2: In vivo shRNA competition

Target gene	mRNA knockdown by shRNA	% Subpop reduction by dox*	Primer Sequence
Transketolase	Yes	-5.8	GGAAGATTAGTTCTGACTTGG (SEQ ID NO:28)
Transketolase	No	-13.5	GGATGCTATTGCGCAGGCTG (SEQ ID NO:29)
K-Ras	Yes	96.8	GCGATATAGCTAGTTCAGGAT (SEQ ID NO:30)
K-Ras	No	-22.5	GGATAGCCAACAATAGAGGTAAA (SEQ ID NO:31)
Ceramide Kinase	Yes	-187.2	ATAGTACGCTCCTTCGCTATTC (SEQ ID NO:32)
Ramp2 (Hairpin 1)	Yes	99.4	GTGAGTCTCAAAGATGATCC (SEQ ID NO:33)
Ramp2 (Hairpin 2)	Yes	99.0	ACTGTCTTTACTCCTCCATAC (SEQ ID NO:34)
PTK7 (Hairpin 1)	Yes	95.8	CTTGATGTTGCAGCTGTTGC (SEQ ID NO:35)
PTK7 (Hairpin 2)	Yes	88.0	CACTTTCAGCAATATTGGCC (SEQ ID NO:36)
Luciferase	Yes	-323.6	GCTCTCGCTGAGTTGGAATC

			(SEQ ID NO:37)

*subpop = subpopulation; dox = doxycycline

In the experiment summarized in Table 3 (below), HCT116/Tet repressor cells individually expressing a dozen shRNAs against genes of interest were cultured in vitro separately and then mixed in equal ratio at concentration of 10^7 cells/ml. The mixed population of cells was mixed with Matrigel™ (1:1) and injected into 20 SCID mice (10^6 cells/0.2 mls per injection, 2 injection sites per mouse). The mice were then divided into two groups: one group received doxycycline at day 6 to induce the expression of shRNAs and the other group did not.

The tumors were collected at day 21. The average weight of the tumors without doxycycline treatment was 0.329g (n=28) and that with doxycycline treatment was 0.356g (n=27). Upon pulverization of the tumors, the DNA was extracted (Qiagen). Equal amount of DNA from tumors with or without doxycycline treatment was pooled into two separate samples. Quantitative PCR with SYBR green (Qiagen) was used to examine the abundance of the shRNA hairpins using a vector specific primer, pLentiTO2B_1 CTCGACGGTATCGCTAGTCC (SEQ ID NO:38), and a hairpin-specific primer (listed in Table 3) in the two groups of DNA. During the PCR cycle, the polymerase was first activated at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C, annealing at 54°C and elongation at 72°C.

The representation of the cells expressing each individual shRNA in the tumor samples was calculated. In this experiment, the abundance of the cells expressing PTK-7 hairpin 1 or 2 was decreased by 92.6% or 58.1% respectively in tumors exposed to doxycycline compared to that in untreated tumors.

Table 3: In vivo RNAi competition

Target gene	mRNA knockdown by shRNA	% Subpop reduction by dox*	Primer Sequence
c17orf26	Yes	98.6	CCGTAGAGCCGAAGTTCAGT (SEQ ID NO:39)
HAS3	Yes	78.1	GAGAATGTTCCAGATGCGGC (SEQ ID NO:40)
G6PD + Transketolase	Yes	19.8	GATGTCGGATGCACACATATTA (SEQ ID NO:41)

Transketolase	Yes	-72.2	GGAAGATTAGTTCTGACTTGG (SEQ ID NO:42)
Transketolase	No	7.2	GGATGCTATTGCGCAGGCTG (SEQ ID NO:43)
K-Ras	Yes	91.1	GCGATATAGCTAGTTCAGGAT (SEQ ID NO:44)
K-Ras	No	23.5	GGATAGCCAACAATAGAGGTAAA (SEQ ID NO:45)
Ramp2 (Hairpin 1)	Yes	98.1	GTGAGTCTCAAAGATGATCC (SEQ ID NO:46)
Ramp2 (Hairpin 2)	Yes	99.9	ACTGTCTTTACTCCTCCATAC (SEQ ID NO:47)
PTK7 (Hairpin 1)	Yes	92.6	CTTGATGTTGCAGCTGTTGC (SEQ ID NO:48)
PTK7 (Hairpin 2)	Yes	58.1	CACTTTCAGCAATATTGGCC (SEQ ID NO:49)
Luciferase	Yes	-44.7	GCTCTCGCTGAGTTGGAATC (SEQ ID NO:50)

*subpop = subpopulation; dox = doxycycline

Example 10: Antibody Production

The GP153 antigens listed in Table 4 were recombinantly produced as GST fusion proteins and used to obtain rabbit polyclonal antibodies. Crude rabbit sera were tested by conventional ELISA, using a secondary antibody against rabbit IgG. Approximate EC_{50} values obtained with each serum are shown in Table 4 below.

Table 4: Polyclonal Antibodies

Designation	EC_{50}	Amino Acid Sequence
GP153SD1	2×10^{-3}	Amino acids 30-280 of SEQ ID NO:1
GP153SD3	2×10^{-4}	Amino acids 30-280 of SEQ ID NO:1
GP153RW2	2×10^{-3}	Amino acids 236-488 of SEQ ID NO:1

A conventional Protein A purification technique was used to obtain an IgG fraction from the GP153SD1 and GP153SD3 sera. This partially purified polyclonal antibodies were subjected to fluorescence activated cell sorting (FACS) analysis, using cells known to display GP153 on their surface. This analysis

demonstrated that the GP153 polyclonal antibodies were binding specifically to the GP153 extracellular domain.

To generate monoclonal antibodies against GP153SD1, GP153SD3, GP153RW2, and GP153Fc (amino acids 1-704 of SEQ ID NO:1 fused to Fc), BalB/c and AJ mice were immunized with 100ug of each respective antigen, and crude sera were tested by ELISA. Test bleeds were assessed every month, until the titers remained high (EC50 < 10-3). Spleens from mice immunized with GP153Fc were isolated and fused with human FO myeloma cells. The fusion products were plated into 96-well plates and subsequently tested by ELISA. Supernatants from fusion products that were positive by ELISA displayed EC50 values that ranged from 0.1 to <10-3. Fusion products from the positive wells will be further subcloned and tested to generate monoclonal hybridoma lines.

Example 11: In vitro validation of GP153 using shRNA and antibodies

Lentiviral vectors expressing interfering dsRNA against PTK7 were generated to modulate the expression of PTK7 in vitro and in vivo and assess its possible role in tumor maintenance. Lentiviral vectors expressing sequences encoding interfering dsRNA against PTK7 were generated. To target specific regions of a PTK7 mRNA, the following oligonucleotides were used with a primer specific to the U6 small RNA promoter to form double-stranded DNA in a polymerase chain reaction, using a vector containing this U6 promoter as a template. The PCR product was then ligated into a pENTR11 vector (Invitrogen) into which the inducible U6TO2B promoter (described in WO 2004/056964) was cloned. Expression of the insert resulted in expression of a short hairpin RNA. The hairpin structure displayed inhibitory effects on PTK7 expression.

The sequence of the sense-loop-antisense sequence is shown below for each shRNA transcript. Termination of shRNA transcription is caused by addition of four or more Ts after the shRNA sequence. This results in the terminal UU doublet below:

(1) PTK7_1130-1154 shRNA transcript. PTK7 target nucleotides (in boldface)

GGCCAAUGUUGCUGAAAGGGAGCUUCCUGUCAC**UCACUUUCAGCAA UAUUGGCC**UU (SEQ ID NO:51)

(2) PTK7_1996-2020 shRNA transcript. PTK7 target nucleotides (in boldface)

GCAACAGGUGCAACAUCAUGCGCUUCCUGUCAC**GCUUGAUGUUGCA GCUGUUGC**UU (SEQ ID NO:52)

The U6TO2B-PTK7 shRNA expression cassettes generated were subsequently shuttled into the pLenti6 lentiviral vector using LR Clonase (Invitrogen V496-10 and 11791-019). Lentiviruses were generated using Invitrogen's packaging system. Briefly, 6 μg of lentiviral DNA was mixed with 6 μg of packaging mix from the ViraPower lentiviral support kit (Invitrogen K4970-00), and transfected into 293T cells using Lipofectamine 2000 (Invitrogen 11668-027). Supernatants were harvested after 48 hours, and viral titers were estimated by performing infections of HCT116 cells with serial dilutions.

The PTK7 inducible hairpins were introduced into an HCT116 cell line stably expressing luciferase and TetR that was described in WO 2004/056964. Human colorectal carcinoma cells, HCT116, were previously infected with retroviruses containing a luciferase expression construct containing the hygromycin resistance gene. After selection with hygromycin (50 μg/ml), the cells were subsequently infected with lentiviruses expressing a codon-optimized version of TetR (gpTetR), under the control of the PGK promoter. The gpTetR expression construct contains a Zeocin resistance gene. The stably transfected cells were selected with Zeocin (50 μg/ml) and then subcloned. Single clones were tested for adequate gpTetR expression, using real time RT-PCR, and subsequently infected with lentiviral vectors containing two different PTK7 shRNAs under the control of the inducible promoter U6TO2B, or no hairpin (empty vector). Following selection, cells were grown with or without doxycycline, and tested for: (a) expression of PTK7, (b) ability to form colonies on soft agar, and (c) growth kinetics.

Cells were grown for 72 hours with and without doxycycline (1 μg/ml in DMEM, 10% FBS, 1% penicillin-streptomycin, 10 μg/ml blasticidin, 50 μg/ml zeocin and 50 μg/ml hygromycin). Medium was changed every 24 hours. Total RNA was extracted using the RNeasy™ kit (Qiagen 74104), and real-time PCR analysis was performed using SYBR green (Qiagen 204243) with the following

primers: Beta actin primers were used to control for loading: Forwarding 5'-AAATCTGGCACCACACCTTC-3' (SEQ ID NO:53) and reverse 5'-AGAGGCGTACAGGGATAGCA-3' (SEQ ID NO:54). PTK7 primers: forward 5'-CGCCTCCTTCAACATCAAAT-3' (SEQ ID NO:55) and reverse 5'-CTTGCTGCTGACTGTGTGGT-3' (SEQ ID NO:56). Expression of PTK7 was normalized to that of beta actin to control for total RNA loading. Table 5 shows that upon doxycycline induction, expression of PTK7 decreased as contrasted to comparable cells without docycycline, except for the vector control.

Table 5: Relative Expression of PTK7 by Real-Time PCR

Construct	Doxycycline in medium	Expression*
Empty vector	No	100%
Empty vector	Yes	105%
PTK7-1	No	38%
PTK7-1	Yes	7.6%
PTK7-2	No	39%
PTK7-2	Yes	13.9%

*Percentage of empty vector with no doxycycline induction

Cells were grown as described above, and tested by western blot analysis, using a polyclonal antibody raised against PTK7. Equal amounts of lysate, as determined by total protein content, were loaded in each well. A band of expected size (120 kD) was present in all wells, but highly attenuated in samples from cells transfected with a PTK7 hairpin and cultured on doxycycline.

FACS analysis was also performed with the cells described above. Cells were stained with a polyclonal antibody against PTK7 to assess cell surface expression in the different cells with and without doxycycline. Table 6 summarizes results from experiments showing that in the presence of doxycycline, cell surface expression of PTK7 was decreased in cells expressing the PTK7 hairpins, but not decreased in cells transfected with the empty vector.

For the MTT assay, cells were plated in 24-well plates. After 72 hours, supernatant was removed and replaced with fresh media containing 3-4, 5-dimethylthiazol-2-yl-2,5diphenyl tetrazolium bromide (0.5 mg/ml in media). Cells were then incubated for four hours, and lysed in 0.4 N HCl in isopropanol to assess uptake of the chemical by live cells. Low OD_{580} values indicate inability to take up the chemical, as a result of cell death.

Table 6: MTT Assay Results

Construct	Doxycycline in medium	OD_{580}
Empty vector	No	0.78
Empty vector	Yes	0.73
PTK7-1	No	0.94
PTK7-1	Yes	0.63
PTK7-2	No	0.79
PTK7-2	Yes	0.45

Cyquant analysis. Cells were seeded as above (except 1,000 cells cell/cell in a 96-well plate). Media with and without doxycycline were changed every 48 hours. After 96 hours, the media were removed and the plates frozen at -20°C. Cell proliferation was quantified by Cyquant Cell Proliferation Kit™ (Molecular Probes C7026) as follows. The cell plate was thawed at room temperature and 150 μl cyquant solution (according to vendor's instructions) was added to each well. The plate was then incubated at 37°C for 30 minutes, and the signal was recorded on a fluorescent plate reader with excitation at 485 nm and emission at 528 nm. Knockdown of PTK7 levels in the presence of doxycycline decreased cell proliferation in HCT116 cells, with PTK7 being the more effective of the two (Table 7). The empty vector control did not significantly change the rate of cellular proliferation in the presence of doxycycline in either cell line.

Table 7: Cyquant Assay Results

Cell Line	shRNA Construct	Fluorescence*
HCT 116	PTK7-1	65.3 ± 1.1
HCT 116	PTK7-2	33.3 ± 2.9
HCT 116	Empty vector	112.0 ± 2.4

*Fluorescence value from cells grown on doxycycline divided by fluorescence value from cells grown without doxycycline

HCT116-Luc-TetR cells expressing PTK7 hairpins or empty vector were grown in soft agar with and without doxycycline for seven days. The number of colonies was assessed for each sample and condition. Briefly, 2 x 10^3 cells were mixed with top agar mix (2 x DMEM, 10% FBS, and SeaPlaque agarose, for a final concentration of 0.4% agarose) over a layer of bottom agarose (0.8% final agarose concentration). Media were added the next day (plus or minus doxycycline) and replenished every 24 hours. Colonies were counted after six days in culture. The average number of colonies per well is shown in Table 8 below.

Table 8: Colony Formation in Soft Agar

Construct	Doxycycline in medium	Avg. Colony No.
Empty vector	No	103.8
Empty vector	Yes	109.5
PTK7-1	No	83.8
PTK7-1	Yes	9.3
PTK7-2	No	91.3
PTK7-2	Yes	1.7

Upon doxycycline induction, cells expressing either PTK7 hairpin showed a dramatic decrease in number of colonies.

Other embodiments are within the following claims.